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Corynebacterium pseudotuberculosis - Cell Invasion and Intracellular Survival

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For my Mother and my Father, as a thanks for everything.

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1. Summary

Corynebacterium pseudotuberculosis (Cp) is the causative agent of caseous lymphadenitis (CLA), a chronic bacterial disease of sheep and goats characterized by granulomatous inflammation of lymph nodes. CLA is prevalent worldwide and causes considerable economic losses to the sheep and goat industries.

The ability of Cp to persist as a facultative intracellular parasite is considered an important feature in the pathogenesis of CLA. The present study provides the basis for identifying the host and bacterial factors mediating entry of Cp into phagocytic and non-phagocytic cells and evaluates the possible significance of cell invasion in the pathogenesis and epidemiology of CLA. The aims were to investigate the cell tropism of Cp and to characterize the host-pathogen interactions and mechanisms involved in cell invasion by analyzing the *in-vitro* ability of the bacterium to enter murine J774 macrophages and epithelial buffalo green monkey (BGM) cells.

By means of a gentamicin invasion assay and bright field light microscopy, Cp was shown to enter and multiply within macrophages and actively invade epithelial cells. Scanning electron microscopy revealed that the bacterium seemed to adhere to the BGM cells in clusters and induce its uptake via a “zipper-like” mechanism of engulfment. Using a variety of compounds that act on eukaryotic cell structures, the entry process was found to involve various host signal transduction events and the reorganization of the actin cytoskeleton.

2. Zusammenfassung

Corynebacterium pseudotuberculosis (Cp) ist der Erreger der Pseudotuberkulose, einer chronisch verlaufenden, verkäsenden Lymphadenitis bei Schafen und Ziegen, die weltweit beträchtliche wirtschaftliche Verluste verursacht.

Die Fähigkeit von Cp als fakultativ intrazelluläres Bakterium zu persistieren spielt eine wichtige Rolle in der Pathogenese der Pseudotuberkulose. Ziel der Arbeit war die Identifizierung von bakteriellen und Wirt-assoziierten Faktoren, die bei der Invasion von Cp in phagozytierende und nicht-phagozytierende Zellen eine Rolle spielen. Ausserdem sollten mögliche Auswirkungen des intrazellulären Parasitismus auf die Pathogenese und Epidemiologie der Pseudotuberkulose evaluiert werden. Dazu wurden Untersuchungen zum Zelltropismus und zu potentiellen Zellinvasionsmechanismen von Cp mittels *in-vitro* Invasionsversuchen an murinen J774 Makrophagen und epithelialen Buffalo Green Monkey (BGM-) Zellen durchgeführt.

Mit Hilfe eines Gentamicin-Invasionsassays und Lichtmikroskopie konnte nachgewiesen werden, dass sich Cp intrazellulär in Makrophagen vermehren und aktiv in Epithelzellen eindringen kann. Rasterelektronenmikroskopische Untersuchungen zeigten, dass Cp in Aggregaten an Epithelzellen adhäriert und seine Aufnahme über einen sogenannten „zipper-like“ Mechanismus induziert. Versuche unter Einfluss von Inhibitoren eukaryotischer Zellfunktionen zeigten auf, dass zelluläre Signalmoleküle und das Aktin-Zytoskelett an der Invasion beteiligt sind.

3. Introduction

3.1. *Corynebacterium pseudotuberculosis*

Corynebacterium pseudotuberculosis (*C. pseudotuberculosis*) is a small (1-3 µm), Gram-positive, non-motile, rod-shaped bacterium. It is facultative intracellular, non-sporulating, non-capsulated, facultative anaerobic and katalase-positive. Morphologically, it appears pleomorphic, with club-shaped or even coccoid forms, and shows a characteristic palisade or "Chinese letter" arrangement. Grown on blood agar, it forms dry, friable, chalky white colonies with a narrow band of β-haemolysis (Muckle and Gyles, 1981; Dorella et al., 2005). Bacterial growth is sparse initially and benefits from the addition of whole blood to solid media. (Dorella et al., 2005). When grown in liquid media, *C. pseudotuberculosis* tends to form clumps and develops a granular deposit with a surface pellicle, a feature that has been attributed to the high lipid content in the cell wall of the bacterium (Merchant, 1935; Dorella et al., 2005).

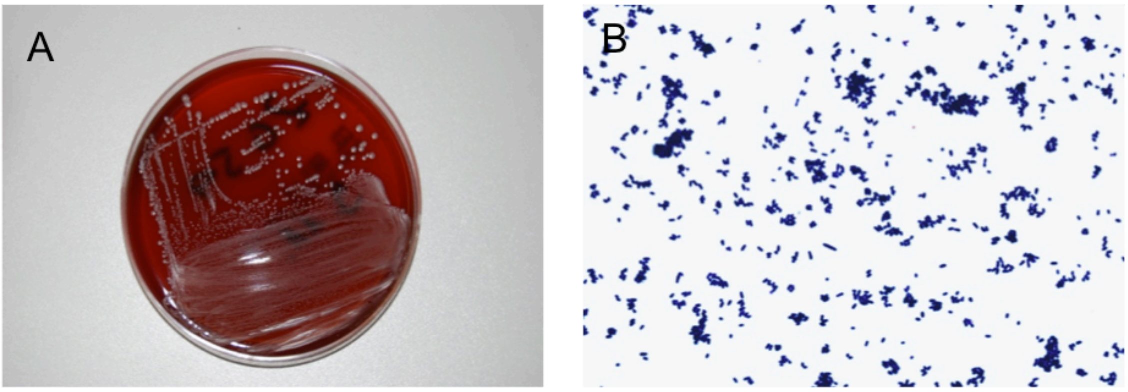


Fig. 1 : *Corynebacterium pseudotuberculosis*
A Chalky white colonies of *C. pseudotuberculosis* grown on sheep blood-containing agar.
B: "Chinese letter" formation of the bacilli in a Gram stain

Taxonomically, *C. pseudotuberculosis* belongs to the genus *Corynebacterium* that is part of the *Actinomycetales* group, which also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus*. The members of this heterogeneous group share certain characteristics, such as a specific cell wall organization and high guanine and cytosine content on the genome. The cell walls of these genera are composed of a huge polymer complex of peptidoglycan and arabinogalactan and are very rich in complex lipid components. The best characterized of these lipids is a long-chain 2-branched 3-hydroxy fatty acid, commonly known as mycolic acid. The mycolic acids of the corynebacteria, also known as corynomycolic acids, are the shortest of the group, being between 20 and 36 C atoms in length, whereas those of *Mycobacterium* consist of chain lengths between 60 and 90 C atoms (Brown and Olander, 1987; Dorella et al., 2005). The corynomycolic acids are an important virulence factor of *C. pseudotuberculosis* as they contribute to its high tenacity and its ability to persist as a facultative intracellular parasite (Baird and Fontaine, 2007).

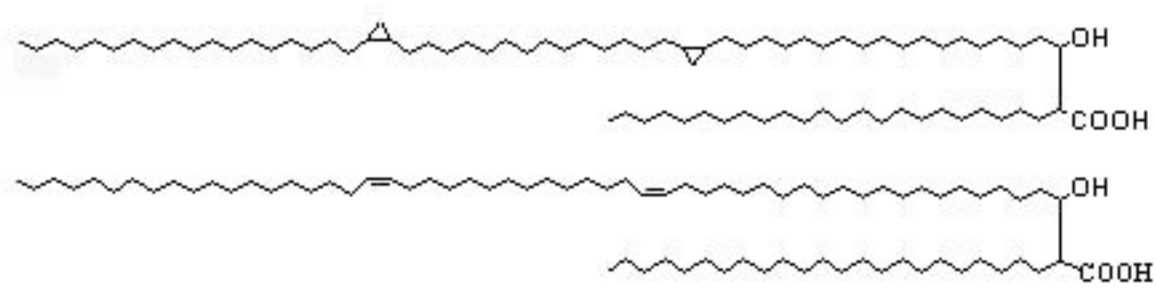


Fig. 2: Chemical structure of mycolic acids
(Picture: www.cyberlipid.org)

Two different biovars of *C. pseudotuberculosis* can be distinguished, based on the different nitrate reduction abilities of the two biotypes. Strains isolated from small ruminants tend not to reduce nitrate to nitrite whereas isolates from horses and cattle almost invariably possess a nitrate-reductase and are, therefore, able to reduce nitrate. Thus, Biberstein et al. (1971) suggested the separation into the two biovars *C. pseudotuberculosis* biovar *equi* and *C. pseudotuberculosis* biovar *ovis*.

All strains of *C. pseudotuberculosis* examined to date are able to produce phospholipase D (PLD), a powerful exotoxin that plays an essential role in pathogenesis (Muckle and Gyles, 1983; Brown and Olander, 1987).

3.2. Caseous lymphadenitis

3.2.1. Clinical signs

C. pseudotuberculosis has been isolated from abscesses and other suppurative lesions in a variety of animals, but only in small ruminants, horses and man it has been recognized as the cause of specific diseases (Brown and Olander, 1987). In sheep and goats it is the causative agent of caseous lymphadenitis (CLA), a disease associated with granulomatous, necrotizing inflammation of one or more lymph nodes that leads to chronic abscessation and enlargement of the lymph nodes, loss of overlying hair and eventual rupture of the abscesses and discharge of pus (Baird and Fontaine, 2007).

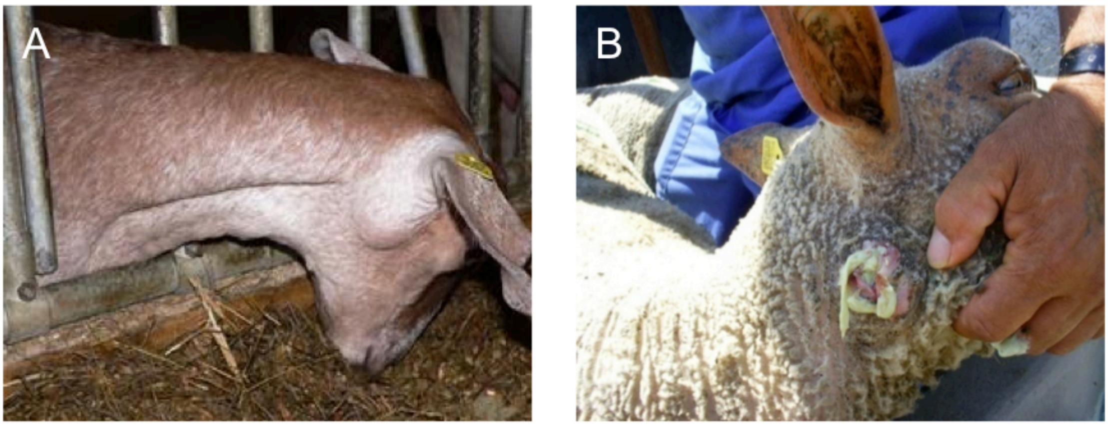


Fig. 3: Clinical signs of CLA.
A: Goat with abscess in parotid lymph node (Picture by Beratungs- und Gesundheitsdienst für Kleinwiederkäuer (BGK), www.caprovis.ch). B: Ruptured abscess discharging yellowish-greenish pus in a sheep suffering from CLA (Picture: IVB-UZH)

The lesions occur in two main forms: the external (or superficial) and the visceral form, which may co-exist within the same animal. The external form is characterized by formation of abscesses in subcutaneous tissue and large superficial lymph nodes such as mandibular, parotid, superficial cervical, subiliac, popliteal or mammary lymph node. The abscesses are enclosed in a firm fibrous capsule and contain thick, greenish-white, odourless material. In sheep, the abscessed lymph nodes often develop a characteristic “onion ring” appearance with concentric bands due to repeated stages of necrosis and capsule formation. The disease

progresses chronically with draining and healing of old abscesses and formation of new lesions, which often recur months to years later in various lymph nodes as the animals are generally not able to totally eliminate the infection. Depending on the size and location of the abscesses, clinical signs such as mastitis can occur. More often, though, the disease progresses subclinically, as the lesions do not appear to greatly affect the well-being of infected animals, and may not be recognized until abscesses are found at slaughter (Batey, 1986c; Brown and Olander, 1987; Williamson, 2001).
The visceral form is associated with abscesses in internal lymph nodes (such as mediastinal, thoracic or lumbar) and organs such as lungs, liver or kidney. Visceral lesions develop from hematogenous spread from regional lesions and are often associated with a more severe form of the disease. Depending on the location of the lesions, clinical signs may be pneumonia, pleurisy or general ill-thrift, i.e. the "thin ewe syndrome", a syndrome associated with weight loss, chronic wasting and a severe decrease in productivity (Batey, 1986c; Brown and Olander, 1987; Pepin et al., 1994a; Williamson, 2001).

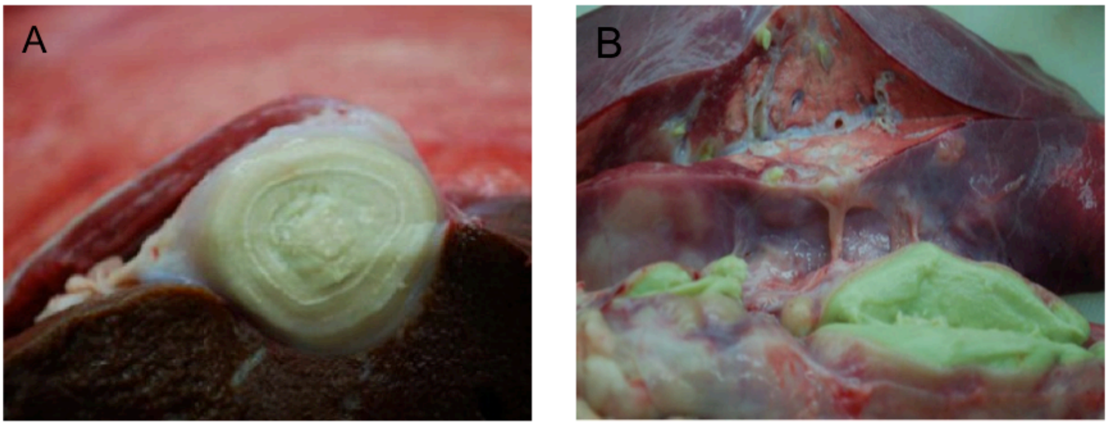


Fig. 4: Visceral form of CLA
A: Liver abscess with characteristic "onion-ring" appearance in a sheep. B: Visceral form of CLA in a sheep: Severe abscessation of lung lymph nodes and pneumonia (Picture: Dr. Joachim Weikel, IVET Innsbruck, http://www.ages.at/ages/gesundheit/tier/pseudotuberkulose/)

In horses, *C. pseudotuberculosis* causes ulcerative lymphangitis, a chronic progressive inflammation of the subcutaneous lymphatic vessels, and was also recognized as the causing agent of deep intramuscular ventral abscesses (Brown and Olander, 1987; Addo, 1983;

Doherr et al., 1998). Zoonotic infections in humans due to *C. pseudotuberculosis* are rare but have been documented occasionally. In the majority of the cases, the patients were occupationally exposed to sheep or goats and developed a suppurative lymphadenitis with a chronic or recurrent course of disease (Blackwell et al., 1974; Mills et al., 1997; Peel et al., 1997; Bregenzer et al., 1997).

3.2.2. Epidemiology and transmission

CLA is endemic in most of the sheep-rearing areas worldwide but shows a particularly high incidence in countries with intensive sheep farming, e.g. Australia or New Zealand, where a prevalence as high as 53% has been reported (Batey, 1986c). Due to the chronic and often subclinical nature of the disease, though, data on incidence and prevalence are often scarce (especially for Europe) and not very reliable. CLA causes significant economic losses to the sheep and goat industries due to wasting and death, carcass condemnation at slaughter, decreases in hide value, wool and milk production as well as reproductive efficiency (Brown and Olander, 1987).

Even though numerous routes of infection (i.e. intradermal, intratracheal, intravenous or intravaginal) have been tried successfully in experiments, it is believed that the transmission of CLA occurs primarily through contamination of superficial skin wounds inflicted during shearing, castration, ear tagging or, especially in the case of goats, through fighting with flock mates or rubbing against contaminated surfaces (Brown and Olander, 1987; Williamson, 2001). Affected animals are thought to be the principal source of contamination as they can shed huge numbers of viable bacteria through purulent discharge of ruptured abscesses or by coughing up discharge from lung lesions (Pepin et al., 1994a; Baird and Fontaine, 2007). Other animals may then be exposed through direct contact with affected animals or via contaminated environment and equipment. Indeed, *C. pseudotuberculosis* has been shown to survive for extended periods in fomites or other organic material as well as in contaminated sheep dipping fluids (Nairn and Robertson, 1974; Augustine and Renshaw, 1986). In goats, traumatized buccal mucosa and uptake of the organism through contaminated feed has been suggested as another possible means of entry, accounting for the greater number of lesions in the head and neck area seen in this species (Brown and Olander, 1987). Due to the high prevalence of pulmonary and thoracic lesions in animals suffering from the visceral form of CLA, a possible role for airborne transmission has been postulated. However, the distribution of the pulmonary lesions indicates a hematogenous or lymphogeneous rather than aerogenous spread (Augustine and Renshaw, 1986). Nevertheless, animals with lung lesions play an important role in transmission as they can contaminate the environment or skin wounds of other animals, especially when they are in close contact (Pepin et al., 1994a). Although Addo (1983) suggested that the common house fly (*Musca domestica*) could act as a mechanical vector transmitting ulcerative lymphangitis in horses, other studies on the role of arthropods and insects as vectors for *C. pseudotuberculosis* were inconclusive (Brown and Olander, 1987).

3.2.3. Diagnosis and disease control

Despite the control of CLA being of major concern to the sheep and goat industry, the eradication of the disease proves difficult because of the limited reliability of diagnostic methods, its poor response to therapeutics, its ability to persist in the environment and the lack of protective vaccines (Williamson, 2001).

For the successful control of CLA, it is first necessary to identify infected animals. Even though the presence of external abscesses in small ruminants is highly suggestive of CLA, a bacteriologic culture of the lesions should be done to exclude other bacterial pathogens such as *Arcanobacterium pyogenes* or *Staphylococcus aureus*. It is usually possible to isolate *C. pseudotuberculosis* from the lesions and the bacterium can be identified with standardized test kits such as the “API Coryne” kit (bioMérieux). Whilst isolation of the bacterium is still considered the gold standard test, its sensitivity is low since it is often difficult to distinguish clinically between infected and non-infected animals, especially in cases without visible lesions. (Baird and Fontaine, 2007). A considerable amount of research has therefore been dedicated to the development of serological tests, including enzyme-linked immunosorbent assay (ELISA), immunoblotting or synergetic haemolysis inhibition test (SHI) test. Most serological tests detect a humoral response to the PLD exotoxin; however, none of these tests are commercially available at the moment, as they have not proved completely satisfactory. False-positive and false-negative results occur, especially in young animals, chronic cases with fibrosed abscesses and animals that have been previously vaccinated. In the Netherlands, an improved indirect double antibody sandwich ELISA, based on purified phospholipase D exotoxin, was used with some success as a tool in disease eradication. A sensitivity of nearly 80% was reported for this test (Pepin et al., 1994a; Williamson, 2001; Baird and Fontaine, 2007).

Although *C. pseudotuberculosis* is sensitive to a range of antibiotics *in vitro* (Muckle and Gyles, 1981; Judson and Songer, 1991), the response to antibiotic treatment *in vivo* is often poor due to the thick capsule around the abscesses and the intracellular lifestyle of the bacterium. Whereas surgical excision in combination with long-term antibiotic treatment has been suggested as a therapy for humans or particularly valuable animals (Bregenzer et al.; 1997), it is commonly accepted that treatment is not a feasible or prudent method of disease control in large flocks. The eradication of the disease through aggressive culling of infected animals and creating of CLA-free flocks is, therefore, considered the more appropriate solution. Additionally, measures should be taken to limit the spread of the disease and prevent new infections, e.g. avoiding of contaminated equipment or sheep dipping fluids and careful testing of new additions to the flock for CLA before housing them with the other animals (Williamson, 2001).

Numerous attempts have been undertaken so far to develop a protective vaccine against CLA. As both, humoral and cell-mediated immunity, seem to contribute to the protection against CLA (Brown and Olander, 1987), several different types of vaccines have been tried. Most of the currently available vaccines are toxoid vaccines, based on inactivated phospholipase D; however, inactivated whole-cell vaccines, live vaccines and DNA-vaccines have also been developed and tested. Even though none of these vaccines provide an overall protection against CLA, they have been shown to limit the spread of the disease and significantly decrease the prevalence in the herd as well as reduce the clinical symptoms in vaccinated animals (Williamson, 2001, Baird and Fontaine 2007).

3.2.4. Situation in Switzerland

According to the Swiss federal veterinary office (FVO), CLA is fairly common in Switzerland. Due to the lack of standardized and commercially available diagnostic tests though, the current prevalence is not known and remains to be investigated (BVET). Regarding the epidemiology, the role of the indigenous game as a possible reservoir for *C. pseudotuberculosis* should be taken into consideration. In Switzerland, herds of sheep and goats are frequently grazed on mountain pastures during summer, often unsupervised by shepherds. In this environment, they could come into close contact with indigenous game, such as the alp ibex (*Capra ibex ibex*) or the alp chamois (*Rupicapra rupicapra*). As cases of CLA have been reported occasionally in alp ibexes and chamois, a reciprocal transmission of *C. pseudotuberculosis* between wild and domestic animals is to be expected and would have to be taken in account if the disease was to be eradicated (Ryser-Degiorgis, 2004). Since the FVO has classified CLA as a monitored disease, confirmed cases have to be reported to the FVO and vaccination of sheep ad goats against CLA is prohibited. Consequently, there are currently no vaccines registered for use in Switzerland (BVET). However, to limit the spread of CLA between and within the herds the Beratungs- und Gesundheitsdienst für Kleinwiederkäuer has established a voluntary monitoring programme for goats. Farmers can subject their flock to annual controls in order and separate or cull infected animals. This strategy allows farmers to built up CLA-free herds and acquire a "clinically free of CLA" status for their flock, a declaration that should prevent other farmers from buying animals harbouring *C. pseudotuberculosis* (http://bgk.caprovis.ch).

3.3. Pathogenesis of *C. pseudotuberculosis*

The pathogenesis of CLA in sheep and goats is still poorly understood. To date research has focused on two known virulence factors of *C. pseudotuberculosis*: the phospholipase D and the mycolic acids.

Phospholipases are glycophospholipid-hydrolyzing enzymes that play an important role in signal transduction and the inflammatory response in eukaryotic cells. Many bacterial pathogens produce phosphilipases as a part of their invasive strategy, e.g. *Clostridium perfringens* or *Listeria monocytogenes* (Songer, 1997). *C. pseudotuberculosis* is able to produce phospholipase D (PLD), a phosphatidylcholine phosphatidohydrolase that functions as a sphingomyelinase, catalyzing the dissociation of sphingomyelin into ceramide and choline (Pepin et al., 1994a). PLD is known to be an important virulence factor since isolates of *C. pseudotuberculosis* where the *pld* gene has been deleted from the genome are incapable of progressing from the site of infection and do not produce the classic lymph node abscesses (Pepin et al, 1994a). Several biological activities have been reported for PLD, including dermonecrosis, lethality and synergistic lysis of erythrocytes in the presence of *Rhodococcus equi* exotoxin (Pepin et al., 1994a; Baird and Fontaine, 2007). Jolly (1965) showed that PLD functions as a permeability factor that increases the vascular permeability and causes the leakage of plasma from the vessels through hydrolysis of sphingomyelin in endothelial membranes. This effect may assist pathogenesis by allowing the bacteria to disseminate from the site of infection to the local lymph nodes (Brown and Olander, 1987; Pepin et al. 1994a). This hypothesis was supported by Zaki (1976), who showed that antibodies against the toxin prevented the dissemination of the bacterium from the portal of entry. He concluded that the exotoxin, while not involved in the formation of abscesses, was crucial for the spread of the organism. Furthermore, PLD may assist *C. pseudotuberculosis* in avoiding phagocytosis early in infection as it was shown to activate complement, and thus deplete it from the surrounding area, as well as impair the chemotaxis of neutrophils (Pepin et al., 1994a). Since *C. pseudotuberculosis* has the capacity to replicate within and escape from macrophages, other authors have suggested that PLD might also play a role in the escape from the phagosome and macrophage death (Pepin et al., 1994a; McKean, 2007).

The coat of waxy mycolic acids on the cell surface of *C. pseudotuberculosis* plays a major role in pathogenesis. It provides the organism with mechanical and, possibly, biochemical protection. It has therefore been suggested that the mycolic acid coat also enables the bacterium to survive for extended periods within the environment, a feature seen in other members of the *Actinomycetales*, in particular *Mycobacterium* (Baird and Fontaine, 2007). Indeed, *C. pseudotuberculos*is is relatively resistant to environmental conditions and shows a fairly high tenacity in organic material (Augustine and Renshaw, 1986).
However, the cell surface lipid also protects the bacterium from the hydrolytic enzymes within lysosomes and enables it to survive phagocytosis and exist within the host cell as a

facultative intracellular parasite (Hard, 1972; Brown and Olander, 1987; Williamson 2001). Hard (1972) first labelled *C. pseudotuberculosis* as a facultative intracellular parasite. He infected mouse macrophages intraperitoneally with *C. pseudotuberculosis* and examined the ultrastructural changes by electron microscopy. He observed that five minutes after infection, the bacteria were already engulfed by the macrophages and could be found in large vacuoles surrounded by an electron dense layer, which he identified as the cell surface lipid of *C. pseudotuberculosis*. Furthermore, he showed that macrophages that had ingested bacteria underwent rapid degeneration and cell death, releasing bacteria into the extracellular space, whereas the bacteria remained intact. He concluded that *C. pseudotuberculosis* is able to survive within the phagolysosomes and causes the death of its host cells due to the toxic effect of its surface lipid (Hard, 1972; Hard 1975). Tashjian and Campbell (1983) confirmed this hypothesis in an electron microscopy study using caprine mammary macrophages infected with *C. pseudotuberculosis*. They observed that, despite the fusion of macrophage lysosomes with phagosomes containing bacteria, the caprine macrophages underwent progressive degeneration while the bacteria survived.

The capacity to survive and replicate within phagocytic cells is crucial for the pathogenesis of *C. pseudotuberculosis.* It is thought that the organism migrates within macrophages from the initial site of entry to the eventual site of lesion development (Baird and Fontaine, 2007). There is a general consensus amongst researchers that *C. pseudotuberculosis* does not establish a persistent infection at the site of entry but disseminates rapidly, both as free bacteria and within macrophages, to the local lymph nodes (Ayers, 1977; Batey 1986c; Pepin et al., 1991). Pepin et al. (1991) showed that huge numbers of neutrophils were first recruited to the site of infection and began to appear shortly afterwards in the local lymphatic drainage while the number of macrophages in the lesions rose dramatically on the third day after infection. As *C. pseudotuberculosis* is able to survive phagocytosis, the phagocytic cells become the means by which the bacterium gets transported to the local lymph node. Additionally, the PLD exotoxin enhances the transfer of the bacterium via the lymphatics by inducing an inflammatory response and increasing the vascular permeability and lymph flow (Batey, 1986c; Pepin et al., 1991; Baird and Fontaine; 2007). Upon dissemination of the bacterium, it comes to the formation of micro-abscesses in the local lymph node, where, after an initial phase of neutrophil dominance, macrophages infiltrate the lesions (Pepin et al., 1991). Even though the macrophages are the main effector cells fighting

C. pseudotuberculosis, they are not able to eliminate the pathogen; a cycle of phagocytosis, intracellular multiplication and death of the host cells starts, leading to the transformation of the abscesses into epitheloid cell granulomas with a central area of necrosis surrounded by a rim of epitheloid cell and fibrous tissue (Pepin et al., 1991). As seen in other intracellular pathogens, the formation of granulomas results in the encapsulation and entrapment of the lesions but also provides the basis for the chronic nature of the disease. Since the bacteria remain viable in the granumolas, the animals are not able to eliminate the infection and often stay life-long carriers. Occasionally, bacteria escape from the abscesses, possibly through emigration of infected phagocytic cells, and spread further via the blood or lymphatic system to other organs or lymph nodes where they are retained in smaller blood vessels and cause the formation of new lesions (Batey, 1986c, Pepin et al., 1991).

Additionally, the cytotoxic properties of the surface lipid directly contribute to abscess formation; the subcutaneous injection of mycolic acids elicits an inflammatory response, resulting in localized swelling and central haemorrhagic necrosis, and induces sterile pyogenic lesions (Hard, 1975; Ayers, 1977, Baird and Fontaine, 2007). Muckle and Gyles also demonstrated a direct relationship between the quantity of cell surface lipid produced by different strains of *C. pseudotuberculosis* and their ability to induce chronic abscessation (Muckle and Gyles, 1983).

3.4. Phagocytosis and bacterial invasion

Many pathogenic microbes are capable of inducing their own uptake into normally non-phagocytic cells or influencing their fate upon internalization. These so-called “invasive bacteria” actively exploit the host cytoskeleton and the cellular signalling pathways to invade and multiply within their host cells. As bacterial invasion is mechanistically similar to phagocytosis in macrophages/monocytes, the mechanisms of classic phagocytosis should be explained first in order to understand the invasion strategies of bacterial pathogens (Dramsi and Cossart, 1998; Finlay and Cossart, 1997; Cossart and Sansonetti, 2004),

3.4.1. Phagocytosis in macrophages

Cells are able to internalize exogenous material by different mechanisms such as phagocytosis or pinocytosis. Phagocytosis is the uptake of large particles of at least 0.5 μm in diameter whereas pinocytosis refers to the uptake of fluid and solutes. Phagocytosis is known to be dependent on the actin cytoskeleton as it can be blocked by cytochalasin D, a substance that inhibits the actin polymerization. Pinocytosis, on the other hand, requires a clathrin-based mechanism and is usually actin-independent (Aderem and Underhill, 1999; May and Machesky, 2001; Jeng and Welch, 2001). Phagocytosis is an extremely complex process with different molecular mechanisms of internalization, depending on the type of cells, receptors and particles involved. Monocytes/macrophages and neutrophiles are professional phagocytes and very efficient at internalizing particles but most other cells have some phagocytic capacity too. The phagocytic mechanisms, however, differ between the cell types as each cell line possesses distinct cell surface receptors. Additionally, most particles are recognized by more than one receptor, leading to a certain amount of cross-talk and coordination between the receptors and inducing a multiplicity of downstream signals, whose complex interactions and convergence are still poorly understood (Kwiatkowska and Sobota, 1999; Aderem and Underhill, 1999; Underhill and Ozinsky, 2002).

During phagocytosis the cell engulfs exogenous particles through an extension of the cellular membrane, a process driven by the reorganization of the actin cytoskeleton in the region of the plasma membrane that contacts the particle. The polymerization of actin microtubules that is necessary for these membrane protrusions is induced by a signalling cascade, which is initiated through the interaction of plasma membrane receptors with ligands on the surface of the particle (Greenberg, 1995; Kwiatkowska and Sobota, 1999).

The cell surface receptors engaged in phagocytosis can be broadly classified in two groups (Kwiatkowska and Sobota, 1999): the first group recognizes surface components of microbes, the so-called "pathogen-associated molecular patterns" (PAMPs). Mannose receptors, β-glucan receptors, the DEC 205 or scavenger receptors, which recognize surface components on bacteria including LPS, belong to this group (Aderem and Underhill, 1999). The second group of receptors are opsonin-dependent; they recognize opsonins, such as immunoglobulin G (IgG) or complement fragments C3b and iC3b, when they are bound to the surface of pathogens. IgG antibodies bound to foreign particles interact with the cellular Fcγ-receptors

(FcγR) by means of their unoccupied Fc-domain, whereas complement fragments C3b and iC3b bind to the cellular complement receptors (CRs), which belong to the integrin superfamily. (Kwiatkowska and Sobota, 1999)

It was shown that FcγRs form clusters upon cross-linking with specific antibodies or binding of particles. It is therefore assumed that the initial binding of the particles to the cellular membrane induces the clustering of the cell surface receptors, which subsequently initiates the signalling cascade that ultimately leads to the reorganization of the actin cytoskeleton and engulfment (Kwiatkowska and Sobota, 1999). For a complete engulfment, though, the binding of a particle to a single receptor is not sufficient; other receptors have to be progressively recruited to the binding site and ligate the particle surface. Thus, the membrane advances around the particle by continual ligation of new receptors that work in a “zipper-like” manner to engulf the particle (Kwiatkowska and Sobota, 1999). This “zipper-like” uptake of particles is typical for FcγR-mediated phagocytosis. During CR-mediated phagocytosis, on the other hand, the uptake of a particle occurs by a slight variation of this classic “zipper” model. The membrane extensions induced by the binding of a particle to the CRs are much less prominent and the particles are not so much engulfed but instead appear to sink into the cell (Swanson and Baer, 1995).

3.4.2. Signalling pathways in phagocytosis

The internalization of particles is accomplished through simultaneous activation of many signalling pathways that together regulate the phagocytic response. Multiple signalling molecules are involved in phagocytosis, including actin binding proteins, ion channels, kinases and many more. Certain signalling molecules, however, stand out, as they seem to be integration points for the regulation of the phagocytic response. The phosphoinositide 3-kinase (PI 3-kinase), the phospholipase C (PLC), the monomeric GTPases and the proteinkinase C (PKC), for instance, are almost invariably involved in the signalling cascade (Underhill and Ozinksy, 2002).

Tyrosine phosphorylation (TPK) also plays a key role, especially during FcγR-mediated phagocytosis (Kwiatkowska and Sobota, 1999). The binding of IgG to the FcγRs triggers the TPK of certain cytoplasmatic residues of the FcγR named ITAM (immunoreceptor tyrosine-based activation motif). The protein tyrosine kinase responsible for this initial phosphorylation is thought to be a member of the Src family, a family of protein kinases which are anchored in the plasma membrane and are activated upon receptor clustering (Kwiatkowska and Sobota, 1999; Aderem and Underhill, 1999; May and Machesky, 2001). The TPK of the FcγR enables the tyrosine kinase Syk to dock to the receptor, which results in the activation of Syk. Syk is known to be a critical component for FcγR-mediated phagocytosis, but it is still controversial how exactly Syk stimulates the actin assembly. However, it was shown to be concentrated at nascent phagosomes and, supposedly, contributes to the signal transduction through the activation of PI-3 kinase (Kwiatkowska and Sobota, 1999; Aderem and Underhill, 1999; Underhill and Ozinksy, 2002). Syk may activate Cbl, which in turn serves a linker for the PI-3 kinase (Kwiatkowska and Sobota, 1999). Another tyrosine kinase activated by the Src kinases is Vav, a phosphoprotein that serves as a GDP/GTP exchange factor for the monomeric GTPase Rac 1 (see below) (Kwiatkowska and Sobota, 1999).

3.4.2.1. Phosphoinositide 3- kinase

The PI-3 kinase catalyzes the phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3). PI-3 kinase is activated during FcγR-mediated phagocytosis, possibly through Syk kinase and Cbl, but is not required for the initial actin polymerization and the formation of the phagocytic cup since this proceeds normally if the PI 3-kinase is inhibited by wortmannin. It rather appears to control the closure of the phagosome behind the particle or possibly plays a role in the regulation of membrane availability and actin remodelling upon membrane fusion. (Aderem and Underhill, 1999; May and Machesky, 2001; Underhill and Ozinksy, 2002). PI-3 kinase might influence the actin cytoskeleton either by direct cooperation with Rac, one of the monomeric GTPases (see below), or through its products PIP_2 and PIP_3, which can modulate the activity of the actin binding proteins profiling and gelsolin (Kwiatkowska and Sobota, 1999). Furthermore, the PI-3 kinase product PIP_2 is linked to the activation of PKC; PIP_2 is cleaved by PLC, resulting in the release of inositol triphosphate and diacylglycerol which in turn activate PKC family members (Kwiatkowska and Sobota, 1999; Underhill and Ozinsky, 2002).

3.4.2.2. Monomeric GTPases

The proteins of the Rho, Rac, CDc42 and ARF families belong to the superfamily of monomeric GTPases that control various actin-based structures in cells and are key regulators for adhesion, membrane ruffling and stress fiber formation (Underhill and Ozinsky, 2002; Kwiatkowska and Sobota, 1999). The monomeric GTPases have also been found to participate in phagocytosis and a variety of cytoskeletal regulators like phosphotidylinositol 4-phosphate 5-kinase (PIP 5-kinase), myosin II and PIP_2 could be linked to them. Nevertheless, it is not yet defined how exactly the small GTPases regulate the actin structure (Kwiatkowska and Sobota, 1999; Aderem and Underhill, 1999).

3.4.2.3. Protein kinase C

The members of the protein kinase C (PKC) family phosphorylate serin and threonine residues and are activated by the phospholipase products diaglycerol (DAG) and inositol triphosphate (IP_3). PKCs participate in various signalling pathways and are also involved in signal transduction during phagocytosis. CR-mediated phagocytosis, for instance, appears to require PKC activity since the signalling cascade triggered upon activation of the complement receptors is initiated by serin phosphorylation (Kwiatkowska and Sobota, 1999; May and Machesky, 2001). However, controversial results have been obtained for the involvement of PKCs in FcγR-mediated phagocytosis; the signalling cascade induced upon activation of the FcγRs requires tyrosine phosphorylation and PKCs are, therefore, most likely involved in later signalling events or might instead participate in actin remodelling upon internalization and phagosome maturation (Kwiatkowska and Sobota, 1999). Thereby MARCKS (myristoylated alanine-rich C kinase substrate), a major substrate of the PKCs, constitutes a possible link to the actin cytoskeleton as it is an actin cross-linking protein that is deactivated through PKC-dependent phosphorylation. (Aderem and Underhill, 1999; Kwiatkowska and Sobota, 1999)

3.4.3. Complement receptor-mediated phagocytosis

CR-mediated phagocytosis has features in common with FcγR-mediated phagocytosis, such as involvement of the actin cytoskeleton and activation of PI -3 kinase and PLC. However, there are several differences in morphology (as mentioned before) and signal transduction: firstly, CR-mediated phagocytosis only occurs in cells previously activated by various inflammatory cytokines. The activation of the cells triggers the phosphorylation of serine residues in receptor subunits, which leads to a conformational change and clustering of the receptor (Greenberg, 1995; May and Machesky, 2001). CR-mediated phagocytosis is, therefore, dependent on PKC activity whereas TPK is apparently not required (Aderem and Underhill, 1999). Secondly, the activation of the CRs leads to actin filament assembly as well as polymerization of microtubules, which are, unlike in Fcγ-mediated phagocytosis, also needed for the engulfment of the particle (Kwiatkowska and Sobota, 1999).

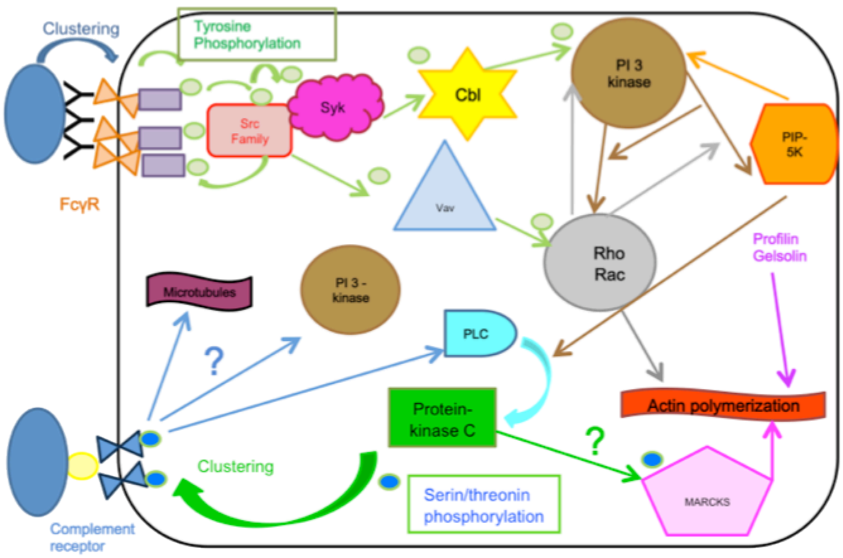


Fig. 5: Signal transduction during Fcγ receptor-mediated phagocytosis.
Interaction of particles with the receptors evokes clustering of the receptors. Kinases of the Src family are activated and tyrosine phosphorylate the receptors residues ITAM. This interaction triggers the activation of the kinases Syk and Vav via tyrosine phosphorylation. Syk and Vav in turn transmit the signal to downstream effectors, such as PI 3- kinase and the monomeric GTP-ases, which induce the reorganization of the actin cytoskeleton. The proteinkinase C might participate in the signalling pathway through its substrate MARCKS, an actin cross-linking protein that is deactivated upon serin/threonin phosphorylation. CR-mediated phagocytosis has features in common with FcγR-mediated phagocytosis, but activation of the complement receptor requires serin/threonin phosphorylation and also involves the polymerization of microtubules.

3.4.4. Bacterial invasion

Invasive bacteria have the capacity to induce their own uptake into normally non-phagocytic cells or influence their fate upon phagocytosis (Dramsi and Cossart, 1998). This strategy allows them to invade their host, avoid the immune response and multiply within a protected niche of the host organism. Furthermore, some invasive pathogens have found methods to replicate within phagocytic cells by either preventing the normal maturation of the phagosome, impair its bacteriolytic activities or by escaping from the phagosome altogether and multiply within the cytoplasm of their host cells (Cossart and Sansonetti, 2004).

The invasion process resembles phagocytosis, but the mechanisms involved in the uptake are specific to each bacterium and, in contrast to phagocytosis, the bacteria play an active role during invasion. Invasive bacteria can express special surface proteins to bind cellular membrane receptors or even interact directly with the cellular machinery to influence the host cytoskeleton. These interactions subsequently trigger a signalling cascade that ultimately leads to the reorganization of the cytoskeleton and uptake of the bacteria (Cossart and Sansonetti, 2004). Invasive bacteria seem to have developed two major mechanisms to induce their uptake: the “zipper-like” and the “trigger-like” mechanism of entry. The “zipper-like” mechanism of entry is similar to classic FcγR-mediated phagocytosis in macrophages (Dramsi and Cossart, 1998); adherence of the microbe leads to the formation of a tight-fitting phagosome that advances around the bacterium by continual ligation of new receptors. In this case, the cytoskeletal rearrangement is only moderate and limited to the area of particle binding. The “trigger-like” mechanism of entry, on the other hand, is similar to membrane ruffling induced in cells by growth factors. Ruffles are unguided membrane pseudopods which can trap extracellular fluid and enclose it in large endocytic vesicles when they fold back against the cell surface, a process known as macropinocytosis. Bacteria and other particles in the vicinity can be engulfed and internalized by these membrane projections. This mechanism is exploited by some invasive pathogens; by sending signals to the cell causing membrane ruffling, they enter the cells almost passively by more or less random capture into forming macropinosomes. In this process, the reorganization of the cytoskeleton is more dramatic and not restricted to the area of binding. The initial signal is therefore sufficient to trigger a response that is not proportional to the size of the particle (Swanson and Baer, 1995).

3.4.4.1. Entry via zipper-like mechanism: *Listeria monocytogenes* and *Yersinia pseudotuberculosis*

Listeria monocytogenes (*L. monocytogenes*), a bacterium capable of entering a variety of cells, possesses two surface proteins that induce the uptake of the bacterium into its host cells via a "zipper-like" mechanism. The surface protein InlA (internalin) interacts with the host E-cadherin, a transmembrane glycoprotein normally responsible for cell-cell interactions at adherens junctions whose cytoplasmatic domains bind catenins, which in turn interact with the actin cytoskeleton (Dramsi and Cossart, 1998; Cossart and Sansonetti, 2004). The second surface protein InlB binds to Met, a transmembrane receptor tyrosine kinase, where it causes the phosphorylation of receptor residues and thus allows the binding of other adaptor molecules such as Cbl (Cossart and Sansonetti, 2004). For both, InlA and InlB-mediated entry, TPK, actin polymerization and PI 3-kinase are required since bacterial internalization is impaired by treatment with genistein (an inhibitor of tyrosine phosphorylation), wortmannin or cytochalasin D (Dramsi and Cossart, 1998).

Yersinia pseudotuberculosis (*Y. pseudotuberculosis*) expresses the outer membrane protein invasin, which mediates entry into cells by binding to cellular β integrins. Integrins are cell surface receptors that participate in cell-cell and cell-matrix adhesion and whose cytoplasmatic tail interacts with the cytoskeleton in focal complexes of adhesion plaques (Cossart and Sansonetti, 2004). Binding of invasin to integrins leads to the formation of a tight-fitting phagosome and internalization of the bacterium through a "zipper-like" mechanism (Dramsi and Cossart, 1998). The invasion process is known to be dependent on the actin cytoskeleton and TPK as internalization of *Y. pseudotuberculosis* is inhibited by treatment with cytochalasin D and genistein (Dramsi and Cossart, 1998). TPK plays a key role in the integrin-mediated signalling pathway, because the two proteins FAK (focal adhesion kinase) and $p130^{cas}$ are tyrosine phosphorylated upon integrin activation and subsequently transmit the signal from the clustered integrins to the cytoskeleton. It is assumed that PI 3-kinase, Src, PKC and Rac also participate in signal transduction, but their definite interactions are still unknown (Rosenshine et al., 1992; Cossart and Sansonetti, 2004).

3.4.4.2. Entry via trigger-like mechanism: *Salmonella typhimurium* and *Shigella flexneri*

The invasion processes of *Salmonella typhimurium* (*S. typhimurium*) and *Shigella flexneri* (*S. flexneri*) are morphologically very similar as both invade into their host cells via a "trigger-like" mechanism of entry. The bacterial effectors that induce the membrane ruffling upon initial contact with the cell membrane are part of the type III secretory system, an apparatus found in many gram-negative bacteria that allows the translocation of effector proteins into mammalian cells (Finlay and Cossart, 1997; Dramsi and Cossart, 1998). In *S. typhimurium,* the genes encoding these effector proteins are known as the *inv-spa* complex, which is located on a chromosomal pathogenicity island. In *S. flexneri,* the *mix-spa* secretion system genes are found on a plasmid-located pathogenicity island (Finlay and Cossart, 1997). These genes encode a variety of proteins, e.g. SipB and SipC in *S. typhimurium* or IpaB and IpaC in *S. flexneri*, which are able to form a pore into the cellular membrane in order to deliver effector proteins directly into the cytoplasm (Cossart and Sansonetti, 2004).

The consecutive signalling events involved in entry differ between *S. typhimurium* and *S. flexneri*. The invasion of *S. typhimurium* is accompanied by increased intracellular calcium concentrations. It seems that the bacterium stimulates the host PLC, which induces the production of IP_3 and thus mobilizes calcium from intracellular stores. Calcium might contribute to the remodelling of the actin cytoskeleton as it controls the activity of a variety of actin binding proteins (Dramsi and Cossart, 1998). Additionally, the *Salmonella* effector protein SipC directly induces actin polymerization upon injection into the cytoplasm (Cossart and Sansonetti, 2004). Other host factors needed for the massive cytoskeletal rearrangement are the small GTPases Cdc42 and Rac 1, which are activated through SopE, another protein of the type III secretory system that acts as an exchange factor for the small GTPases (Danika et al., 1999). TPK, on the other hand, does not seem to be required since tyrosine kinase inhibitors do not prevent the invasion of *S. typhimurium* (Rosenshine et al., 1992). In the case of *S. flexneri*, TPK is required for the invasion as several host proteins become tyrosine phosphorylated upon binding of the bacterium to its host cells, amongst them the actin-associated protein cortactin and the tyrosine kinase Src. (Finlay and Cossart, 1997; Dramsi and Cossart, 1998). The cytoskeletal rearrangement induced by *S. flexneri* is further mediated by the two secreted proteins IpaA and IpaC, which were shown to activate small GTPases Rac1 and Cdc42 and could be associated with the actin-binding protein vinculin (Dramsi and Cossart, 1998; Cossart and Sansonetti, 2004).

3.4.4.3. Entry of *Mycobacterium tuberculosis* into macrophages

Mycobacterium tuberculosis (M. tuberculosis) can enter macrophages, its primary host cells, via a variety of receptors, including mannose receptors, scavenger receptors and FcγRs, which internalize IgG-opsonized bacteria (Aderem and Underhill; 1999). However, the most widely used receptors are probably the CRs. It was shown in several studies that *M. tuberculosis* can bind CRs, either opsonized by complement fragments or non-opsonized, and that CRs are crucial for the internalization of the bacterium (Zaffran et al., 1998; Pieters, 2001; Pieters and Gatfield, 2002). The binding of *M. tuberculosis* to CRs triggers the activation of Rho and Cdc42 and leads to an increase in PLC and TPK activity (Zaffran et al., 1998; Aderem and Underhill, 1999). Internalization through FcγRs, on the other hand, induces the small GTPase Rac and leads to the activation of the macrophage host cell and a stronger inflammatory response compared to CR-mediated entry (Pieters and Gatfield 2002). Additionally, recent work showed an important role for the plasma membrane cholesterol in the uptake of the bacterium. Gatfield and Pieters (2000) found that cholesterol was accumulated at the site of bacterial entry and that depletion of cellular cholesterol prevented the internalization of *M. tuberculosis*. It has been suggested, therefore, that the glycolipid-rich cell wall of *M. tuberculosis* enables it to interact with plasma membrane cholesterol and initiate its own uptake through the so-called “lipid rafts”, cholesterol- and sphingolipid-enriched microdomains in the plasma membrane that participate in polarized secretion, membrane transport and signal transduction (Rosenberger et al., 2000; Pieters, 2001).

3.4.4.4. Invasion mechanisms of *Corynebacterium diphteriae*

Hirata et al. (2004) found that *Corynebacterium diphteriae* (*C. diphteriae*) is able to adhere to human epidermoid larynx carcinoma cells (HEp-2 cells). By means of light microscopy and transmission electron microscopy (TEM) they could show that the bacterium expressed two adherence patterns, the localized and the diffuse pattern of adherence, and that adherence lead to the accumulation of polymerized actin beneath attached bacilli. Using a gentamicin invasion assay, Hirata et al. (2002) proved furher that *C. diphteriae* is not only able to adhere to but also invade HEp-2 cells. Additionally, they examined the effect of cytochalasin E and genistein on the process of invasion and found that both substances significantly decreased the internalization rates, indicating an important role for the actin cytoskeleton and the TPK signalling cascade. Finally, Bertuccini et al. (2004) performed invasion assays under the influence of other compounds that act on eukaryotic cells and found that the invasion of *C. diphteriae* was inhibited by treatment with cytochalasin D as well as monodansylcadaverine (an inhibitor that blocks recycling of receptors to the cellular membrane). Staurosporin (an inhibitor of different protein kinases), on the other hand, lead to an increase of the internalization rates. Furthermore, by examining the bacterium-cell interactions using scanning electron microscopy, they observed that the initial binding of *C. diphteriae* lead to the formation of cellular membrane protrusions that closely attached to the bacteria and lead to the subsequent engulfment und uptake of the pathogen. These findings supported their hypothesis of a receptor-mediated entry and a "zipper-like" mechanism of phagocytosis.

4. Thesis concept

CLA is a chronic bacterial disease of small ruminants that, once endemic in a herd or flock, proves difficult to eradicate and causes considerable financial damage to the sheep and goat industry worldwide. Consequently, most of the more recent research done on CLA has focused on developing protective vaccines or establishing new or more accurate diagnostic tests. Little is known, though, about the pathogenesis and the virulence factors of the etiologic agent *C. pseudotuberculosis*.

The ability of *C. pseudotuberculosis* to persist as a facultative intracellular parasite is considered an important feature in the pathogenesis of CLA. *C. pseudotuberculosis* was shown to enter and survive within macrophages (Hard, 1972), an attribute that allows the bacterium to establish an infection and induce the typical clinical symptoms. The intracellular lifestyle also provides the basis for the chronic nature of the disease as it facilitates the evasion of the host immune response. Macrophages are thought to be the primary host cells of *C. pseudotuberculosis*, but the mechanisms by which the bacterium invades phagocytic cells and prevents its own degradation are not well understood. Furthermore, it remains to be clarified whether *C. pseudotuberculosis* also possesses the ability to invade non-phagocytic cells, a feature seen in other invasive pathogens, e.g. *L. monocytogenes*.

The specific aims of this doctoral thesis were, therefore, to investigate the molecular mechanisms involved in the uptake of *C. pseudotuberculosis* into macrophages and to research the cell tropism of the bacterium by evaluating its capacity for invading epithelial cells. Additionally, the host-pathogen interactions and the possible mechanisms employed by the bacterium to induce its uptake into non-phagocytic cells were analyzed.

The cell tropism and invasion mechanisms of *C. pseudotuberculosis* were investigated by examining the *in-vitro* ability of the bacterium to enter epithelial buffalo green monkey kidney cells (BGM cells) and murine J774 macrophages. By means of a gentamicin invasion assay, bright field light microscopy and scanning electron microscopy, the invasive capacity and the bacterium-host interactions were analyzed. To evaluate the route of entry, the dependence of the bacterial internalization on the host cytoskeleton and different signalling molecules was tested by using a variety of compounds that act as inhibitors on eukaryotic cells.

5. Materials and Methods

5.1. Bacterial strains and growth conditions

Two field isolates of *C. pseudotuberculosis* biovar *ovis* were used for invasion experiments; both obtained from naturally infected CLA-diseased animals:

- Isolate no. 30, sample no. 455, year 1999, sheep
- Isolate no. 54, sample no. 1687(2), year 2000, goat

The bacteria were grown on Columbia blood agar (Oxoid, Basel, Switzerland) at 37°C in a 5% CO_2 humidified atmosphere for three days before used. Subcultures were performed weekly on blood agar to refresh the cultures. Gentamicin at a concentration of 100 µg/ml was bactericidal for both strains.

To obtain homogenous suspensions of *C. pseudotuberculosis*, specialized methods for growth and handling of the bacterial cultures were developed. *C. pseudotuberculosis* aggregates to clumps, if suspended in aqueous media, and it is difficult to achieve a homogenous single cell suspension under standard culture conditions (Batey, 1985). As counting of cluster-forming bacteria leads to less accurate results (Barbosa et al., 1995), special procedures for cell separation had to be employed. The bacteria were, therefore, grown in TSB (Trypticase Soy Broth, Becton Dickinson AG, Allschwil, Switzerland) supplemented with the non-ionic surfactant Tween 80 (f.c. 0.05%, Sigma Aldrich, Buchs SG, Switzerland) to reduce the cell aggregation. The cultures were prepared the day prior to assays and grown overnight at 37 ° C with mild agitation till exponential phase (Batey, 1985). Consequently, all phosphate buffered saline (PBS, bioMérieux, Geneva, Switzerland) used in experiments was supplemented with 0.05% of Tween 80 (PBS-Tween) to avoid renewed clumping of the bacteria. Negative effects of Tween 80 on the cell cultures could not be detected as analyzed by comparative cell culture experiments with and without Tween 80.

The overnight bacterial cultures were washed three times with PBS-Tween, centrifuged at 1500 xg and resuspended in 30 ml PBS-Tween. To ensure a better separation of the bacteria, the cultures were sonicated on ice in a bath type sonicator for 10 min at 37 KHz (McKean et al., 2005, McKean et al., 2007), before adjusting them spectrophotometrically at 600 nm to an optical density OD_{600} = 0.1 nm in order to achieve a final concentration of 5 x 10^7 CFU/ml. The correlation between the optical density and the concentration was prechecked by plate count method; different overnight cultures were set to OD_{600} = 0.1, serially diluted in PBS and plated on Trypticase Soy Agar (Becton Dickinson AG). The agar plates were incubated at 37°C with 5% CO_2 for 48 hours at least before the colonies were counted.

5.2. Cell lines and tissue culture

The mouse macrophage cell line J774 (ATCC no TIB-67^{tm}, LGC Standards, F-67123 Molsheim Cedex) was cultivated in RPMI tissue culture medium (Biochrom AG, Berlin, Germany, purchased by Oxoid) supplemented with 10% inactivated fetal calf serum and 0.1% gentamicin (Biochrom AG). Cultures were grown in a 5% CO_2 humidified atmosphere at 37 ° C. The cells were passaged at a ratio 1:4 once or twice a week.
Buffalo green monkey cells (BGM cells, LGC Standards) were used as an epithelial cell line. The BGM cells were grown in minimal essential medium (MEM, Biochrom AG) supplemented with 5% of inactivated fetal calf serum, 0.1 % gentamicin, 1% non-essential amino acids, 1.4 % HEPES-buffer (1M), 1% vitamins and 1% glutamine (all from Biochrom AG) at 37 ° C. The cells were passaged once a week using a trypsin solution to detach adherent cells. For invasion assays, the BGM cells were transferred to RPMI supplemented with 10% inactivated fetal calf serum (RPMI-FCS) and grown as described for macrophages.

5.3. Gentamicin Invasion Assay

For measurement of invasion and intracellular survival of *C. pseudotuberculosis*, an invasion assay based on the gentamicin invasion assay described by Elsinghorst (1979) was performed. The gentamicin invasion assay is used to evaluate the ability of pathogenic microbes to invade eukaryotic cells. Gentamicin does not penetrate the eukaryotic cell membrane and internalized bacteria are, therefore, protected from the bactericidal effects of the antibiotic while the extracellular bacteria are killed by the gentamicin. This allows an exact quantification of the number of internalized bacteria.

In preparation for the gentamicin invasion assay, the macrophages and BGM cells were split 24 hours prior to the assays. 5 ml of the cell suspension were diluted in 20 ml of RPMI supplemented with 5 % of inactivated fetal calf serum (RPMI-FCS 5%) and seeded on 6-well tissue culture plates (Greiner BIO one, Switzerland, purchased by Huber & Co, Switzerland). By adding 2 ml of diluted cell suspension to each well, the macrophages were seeded at a rate of 3 x 10^6 cells per well while the BGM cells were seeded at a rate of approximately 1 x 10^7 cells per well. Both cell lines were grown to monolayers for 24-48 hours at 37 ° C in a humidified atmosphere containing 5% CO_2.

The cell monolayers, grown to about 75% confluence, were washed two times with PBS before infection with fresh overnight bacterial culture that was treated as described above. For the macrophages, an infection solution was prepared by adding 3 ml of bacterial culture to 12 ml of RPMI-FCS 5%. 1 ml of this solution was added to each well in order to achieve a multiplicity of infection (MOI), i.e. bacteria-to-cell ratio, of approximately 10. The BGM cells were infected with a MOI of 100. Thus, the overnight culture was set to an OD_{600} = 0.5 nm instead of 0.1 nm, which yielded a concentration of approximately 2 x 10^8 CFU/ml. 10 ml of this culture were diluted in 5 ml of RPMI-FCS 5%, before adding 2 ml to each well. Following infection, the cell monolayers were incubated for 30 minutes at 37 ° C in a 5% CO_2 humidified atmosphere (T_0).

30 minutes post infection, the cells were washed two times with PBS to remove unattached bacteria. The first sample was taken at this time point (T_1) before the monolayers were further incubated with fresh RPMI-FCS 5% containing 100 µg/ml of gentamicin (Sigma-Aldrich) to kill the extracellular bacteria. Two hours post infection (T_3), the gentamicin was removed and the cells were futher incubated with fresh RPMI-FCS 5% for a total of 24 hours.
Samples were taken at different time points: 30 minutes (T_1), 1 hour (T_2), 2 hours (T_3), 4 hours (T_4) and 24 hours (T_5) post infection. At each time point, the sampled monolayers were washed two times with PBS before 1 ml of 0.1% Triton X-100 (Sigma-Aldrich) in

PBS-Tween was added to lyse the cells and release the intracellular bacteria. Adherent cells were detached from the well with a cell scraper. To quantify the intracellular bacteria, the suspension of lysed cells and bacteria was serially diluted in PBS-Tween and plated in duplicate on Colombia blood agar. For each time point ($T_1 – T_5$), at least two dilutions were plated on agar plates and each dilution was plated in duplicate. The agar plates were incubated at 37 ° C in a 5 % CO_2 humidified atmosphere for 2 days before the colonies were counted. To calculate the number of viable intracellular bacteria at a time point, the mean number +/- SD (standard deviation) of CFU recovered from the agar plates at this time point was determined. The experiments were repeated at least 5 times for each cell line and the mean of each time point was expressed as CFU/ml and as a percentage of the inoculum.

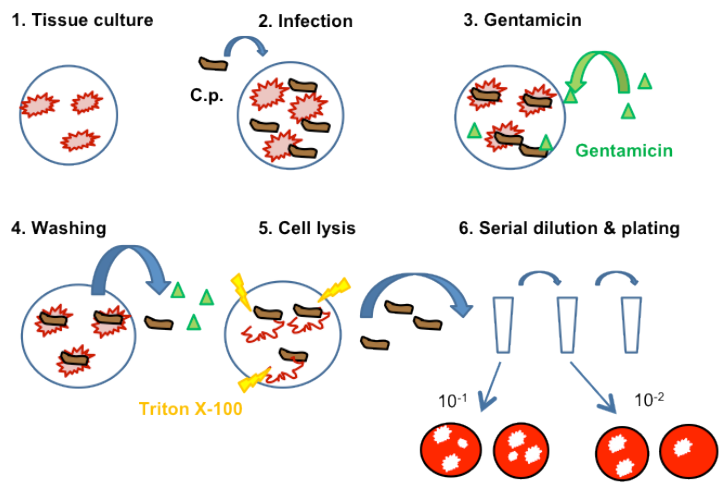


Fig. 6: Gentamicin Invasion Assay
The gentamicin invasion assay is used to quantify the invasion of bacterial pathogens into cells. As the antibiotic gentamicin does not penetrate the cell membranes, bacteria that have invaded the cells are protected from the effects of the antibiotic and can be quantified, e.g. by using serial dilutions and plate count method.

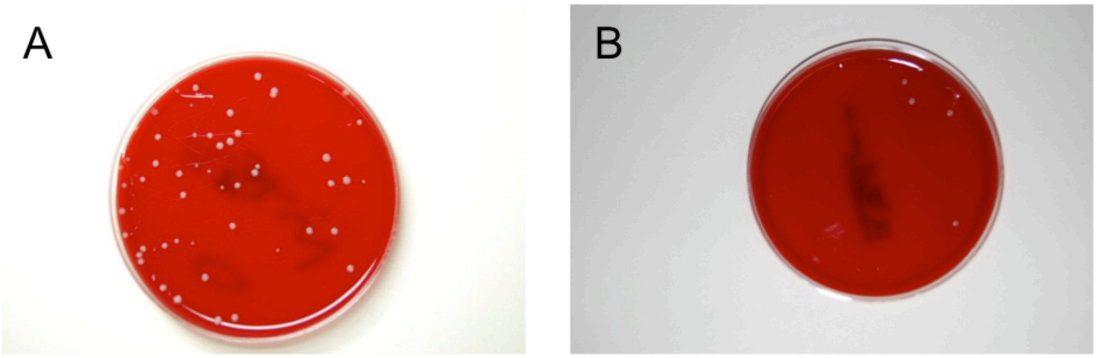


Fig. 7: Plate count method
To quantify the intracellular bacteria, the suspension of lysed cells and bacteria is serially diluted in PBS-Tween and each dilution is plated on blood agar plates in duplicates. After 48 hours of incubation, the colonies are counted. A: Colonies of *C. pseudotuberculosis* on a sheep blood-containing agar plate (undiluted bacterial suspension). B: 1:10 dilution (10^{-1}) of the bacterial suspension.

5.4. Bright Field Light Microscopy

For the bright field light microscopy, the macrophages and BGM cells were grown on sterile round glass coverslips (14 mm) in a 24-well tissue culture plate (Greiner Bio one). The cells were let grown to confluence overnight before a gentamicin invasion assay was performed as described previously. At different time points (T_1-T_5), the sampled monolayers were washed two times with PBS and fixed with methanol for 5 minutes. The coverslips were stained by May Gruenwald-Giemsa and Gram and analyzed with an Olympus BX51 microscope.

Protocol for Gram stains:

1. The cells were covered with cristal violet stain (bioMérieux) for 3 minutes.
2. The cristal violet was removed before Grams iodine solution was added for 2 minutes.
3. The coverslips were decolourized briefly with 100% ethanol and washed gently under the running tap.
4. Saffranin solution (bioMérieux) was added for 15 seconds to counterstain the cells.
5. The saffranin solution was washed away before the coverslips were removed from the tissue culture plate and glued to standard glass slides for microscopy.

Protocol for Giemsa stains:

1. May Gruenwald solution (Sigma Aldrich) was added to the fixed cells for 5 minutes.
2. The May Gruenwald solution was removed with a serological pipette and the cells were covered with PBS set to pH 6.8.
3. After 1.5 minutes, the PBS was removed and Giemsa solution (Sigma Aldrich), diluted 1:20 in PBS, was added to the coverslips and left for 20 minutes.
4. The Giemsa solution was removed and the coverslips were washed briefly in deionized water before they were placed onto glass slides for microscopy.

To investigate whether *C. pseudotuberculosis* actively induces its uptake into the cells, a gentamicin invasion assay was performed with formalin-inactivated bacteria. Macrophages and BGM cells were grown on glass coverslips and infected with formalin-inactivated corynebacteria. The coverslips were stained by May Gruenwald-Giemsa and analyzed with an Olympus BX51 microscope. For the inactivation of *C. pseudotuberculosis,* an overnight culture was prepared and treated as described before. The bacteria were resuspended in 100 ml of PBS-Tween and set to an OD_{600} = 1.0 nm before formalin was added to a final concentration of 0.22% (v/v). The culture was incubated at 37° C with mild agitation for 24 hours. After 24 hours, the culture was washed three times with PBS and resuspended in PBS-Tween. To confirm complete killing, 100 µl of this culture was plated on a blood agar plate and incubated at 37° C in a CO_2 humidified atmosphere for 3 to 4 days. The plate was monitored daily for growth of *C. pseudotuberculosis* and, if necessary, the protocol was repeated until no further growth occurred.

5.5. Scanning Electron Microscopy (SEM)

For the scanning electron microscopy (SEM), the macrophages and BGM cells were grown on carbon-coated glass coverslips in a 24-well tissue culture plate (Greiner Bio-one). The cells were infected with an overnight culture of *C. pseudotuberculosis*, set to an OD_{600} = 0.5 nm as to achieve a MOI of approximately 50. A gentamicin invasion assay was performed with modifications: the BGM cells were incubated for 30 minutes at 37° C in a 5% CO_2 humidified atmosphere, before fresh medium containing 100 µg/ml gentamicin was added. The cells were further incubated for another 60 minutes before the antibiotic was removed. At different time points (T_1-T_5) post infection, the cells were washed two times with PBS and fixed with 2.5% glutaraldehyde by adding 0.5 ml of PBS to each well before slowly dripping in 0.5 ml of 5% glutaraldehyde (glutaraldehyde 5% was prepared by diluting 5 ml of 50% glutaraldehyde (Carl Roth GmbH + Co KG, Karlsruhe, Germany) in 45 ml of PBS). 60 minutes after the fixation, the glutaraldehyde was removed. The cells were washed two times with PBS and covered with PBS.
As the uptake of *C. pseudotuberculosis* into the macrophages occurred very fast, the time points for the fixation had to be chosen differently. At 5 minutes, 10 minutes, 20 minutes, 30 minutes and 60 minutes post infection, the cells were washed two times with PBS and fixed with 2.5% glutaraldehyde according to the protocol for the BGM cells.

Following the fixation with glutaraldehyde, the cell samples were postfixed with 2% osmiumtetroxide in PBS for 30 minutes. The cells were washed three times with aqua bidest and dehydrated in graded series of ethanol (i.e.: 25-50-75-90-95-100% ethanol for 5-15 min each). After washing the samples twice in water-free absolute ethanol, they were transferred to a critical point dryer (BAL-TEC CPD 030 critical point dryer; Balzers, Liechtenstein) under dry ethanol. The medium of dry ethanol was changed against fluid CO_2 at about 10° C for approximately ten times before the samples were dried above the critical point of CO_2 at 31° C and 73.8 bar. The gas was slowly released at 40° C and the dried samples were mounted on SEM stubs with carbon paste or carbon pads. Finally, the samples were sputter coated with 10 nm of platinum using the BAL-TEC MED 020 coating system and analyzed with a Zeiss Supra 50 VP scanning electron microscope.

5.6. Inhibition of *C. pseudotuberculosis* invasion

To investigate the molecular mechanisms involved in the entry of *C.pseudotuberculosis* into macrophages and BGM cells, the cells were treated with different substances that act as inhibitors of distinct eukaryotic cell functions. The inhibitors were added to the cell monolayers before a gentamicin invasion assay was performed as described previously (see chapter 5.3.).

Stock solutions were prepared of all inhibitors (all purchased at Sigma-Aldrich) at the following concentrations:

Wortmannin	1 mM in DMSO
Genistein	10 mM in DMSO
Staurosporin	1 mM in DMSO
Cytochalasin D	10 mM in DMSO
Monodansylcadaverine	20 mM in DMSO
Colchicine	10 mg/ml in PBS
Sodium *ortho*-vanadate	100 mM in deionized water set to pH 10

For the gentamicin invasion assays, the stock solutions were diluted in RPMI-FCS 5% at the following concentrations: wortmannin (2 µM), genistein (100 µM), staurosporin (2 µM), cytochalasin D (100 µM), monodansylcadaverine (200 µM), sodium *ortho*-vanadate (100 µM) and colchicine (10 µg/ml). 30 minutes prior to infection, the inhibitors were added to the cell monolayers and maintained throughout the whole assay. An untreated 6-well tissue culture plate was infected simultaneously in each gentamicin invasion assay as a negative control. To assess the effect of the inhibitors on the level of bacterial internalization, the number of intracellular *C. pseudotuberculosis* recovered at 2 hours post infection was compared between treated and untreated cells. All experiments were repeated at least three times. Inhibitors that had shown a significant effect on the internalization rates of *C. pseudotuberculosis* were subsequently added to the cells at different concentrations in order to investigate whether the inhibitory effect was dose-dependent.

All inhibitors were tested for possible effects on cellular and bacterial viability by microscopic examination and CFU counting of treated and untreated samples respectively.

6. Results

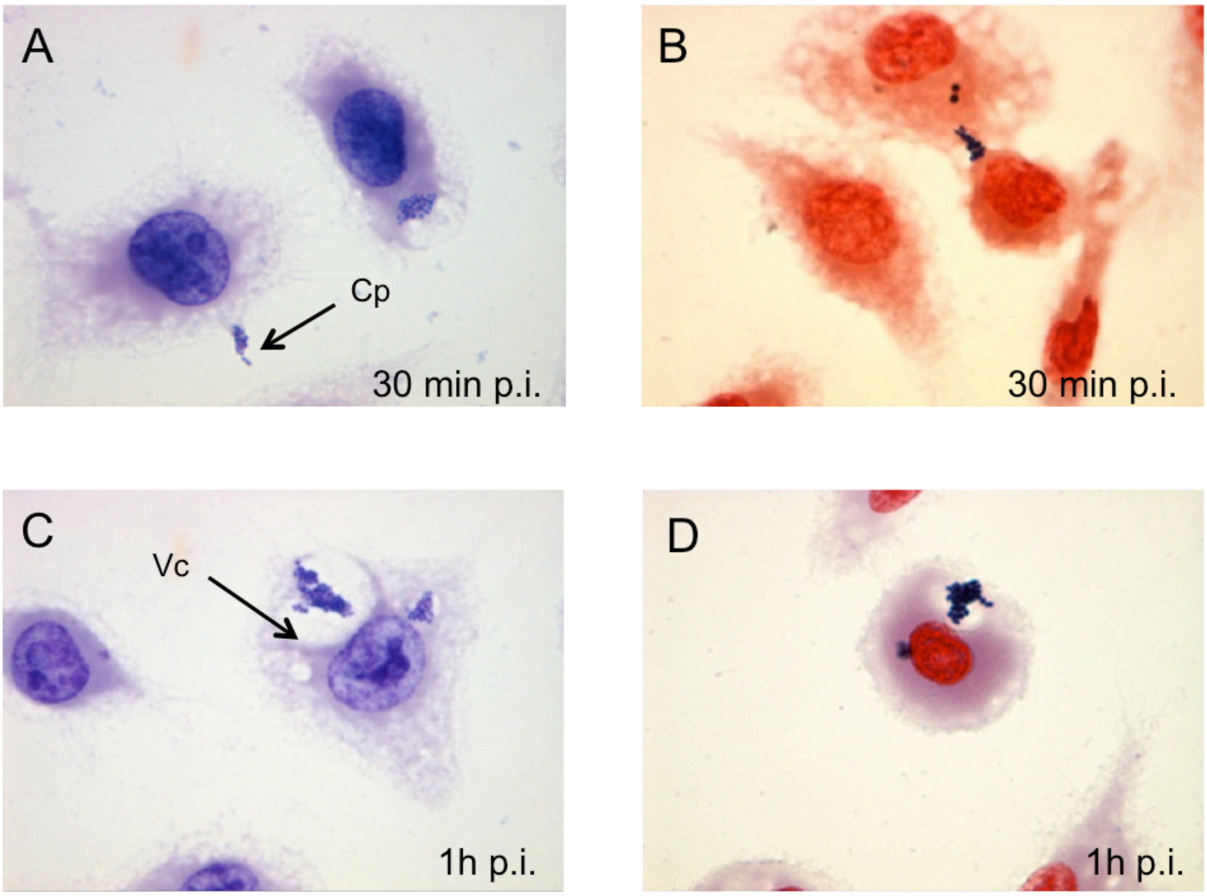
6.1. Bright Field Light Microscopy

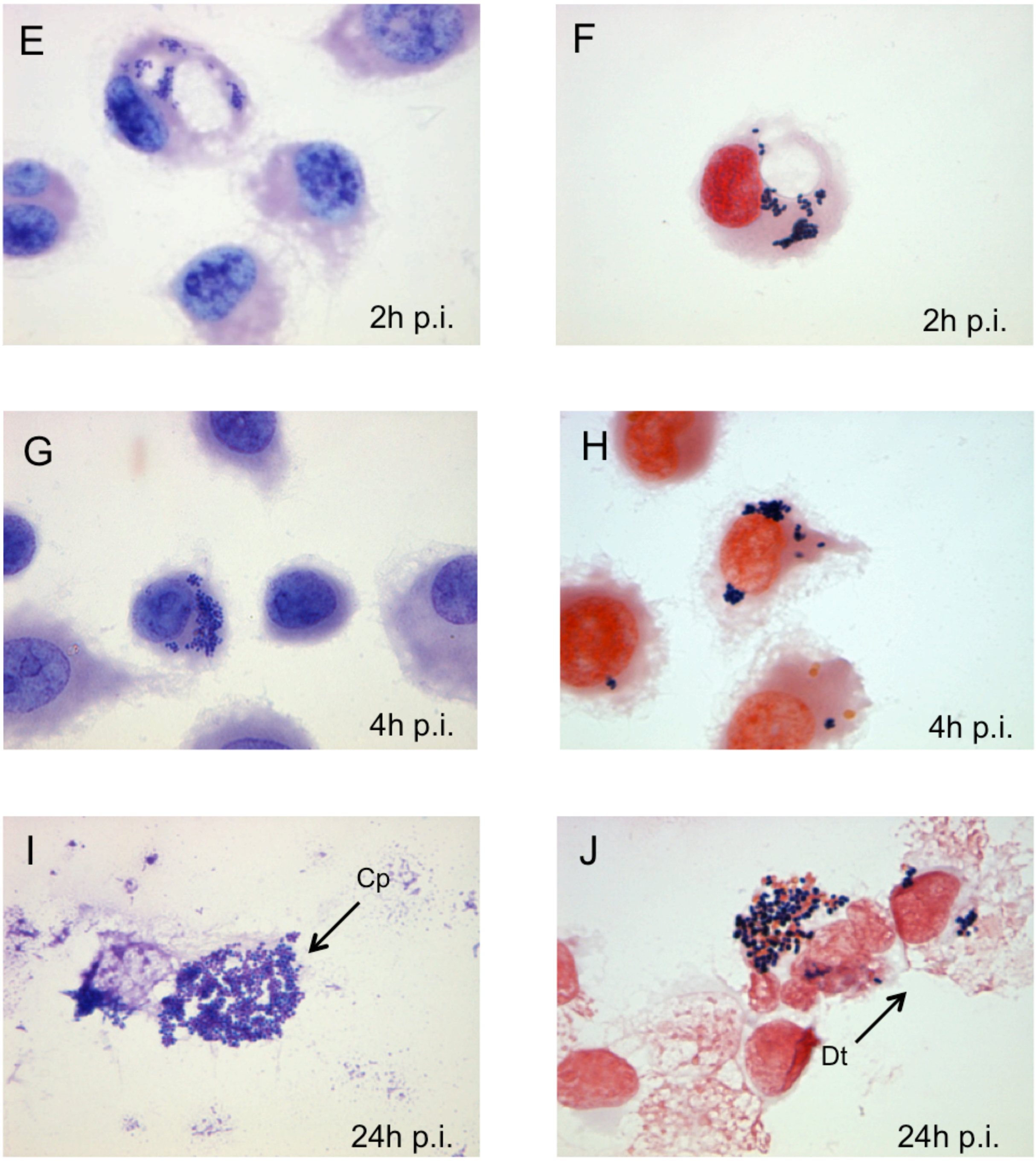
Light microscopy was used to evaluate the ability of *C. pseudotuberculosis* to invade and survive within cells as it is a useful and rapid method to visualize bacteria and cells. The macrophages and BGM cells were infected with *C. pseudotuberculosis* before a gentamicin invasion assay was performed (see chapter 5.3.). Gentamicin was added to the cells to kill the extracellular bacteria and ensure that only intracellular bacteria were detected. In order to observe the progression of the internalized bacteria, the cells were fixed with methanol at different time points post infection and stained by Gram and May Gruenwald-Giemsa. The Gram stain allowed an easy distinction between the bacteria and the cells whereas the cell structure was discernable in more detail when stained by May Gruenwald-Giemsa.

6.1.1. Microscopy of the macrophages infected with *C. pseudotuberculosis*

The mouse macrophage cell line J774 was chosen for the invasion assays since *C. pseudotuberculosis* is known to survive within macrophages (Hard, 1972). The macrophages appeared as large, pleomorphic cells with a kidney-shaped nucleus and a vast cytoplasm that extended into star-like appendages, giving the cells their characteristic shape. When stained by Gram, the cells were of a reddish-pink colour while May Gruenwald-Giemsa stained macrophages appeared bluish-purple with the nucleus being distinguishable by its darker blue colour. Being a Gram-positive bacterium, *C. pseudotuberculosis* was stained blue, in both Gram and Giemsa stains, and could be detected as small coccoid bacilli generally aggregated to small clumps of about 10-50 bacteria.

As shown in Fig. 8, the bacteria had already invaded the macrophages after 30 minutes of infection and were visible in the periphery of the cytoplasm, often associated with cellular protrusions that seemed to have been extended towards them by the macrophages. The uptake of the corynebacteria occurred very fast; 15 minutes after the infection, the bacteria were found mostly inside the cells while only few extracellular bacteria could be detected. Since *C. pseudotuberculosis* tends to form clumps, only 70-80% of the macrophage population was invaded by multiple bacteria, which clustered together at the site of entry. 1 hour after the infection, the bacteria could be observed within large cytoplasmatic vacuoles, from where they seemed to escape progressively into the cytoplasm after 2 hours of infection. After further 2 hours of incubation, the bacteria were found mainly in proximity to the cell nucleus. Finally, at 24 hours post infection, the bacteria had significantly multiplied within the macrophages and caused the degeneration and rupture of their host cells. Cell debris and large clumps of bacteria that had been released into the extracellular space could be seen. A considerable part of the macrophage population had already detached from the glass surface at this time point and nearly 100% of all cells had detached when the macrophages were incubated for a total of 48 hours. The non-infected macrophages appeared healthy and showed a normal morphology.




E
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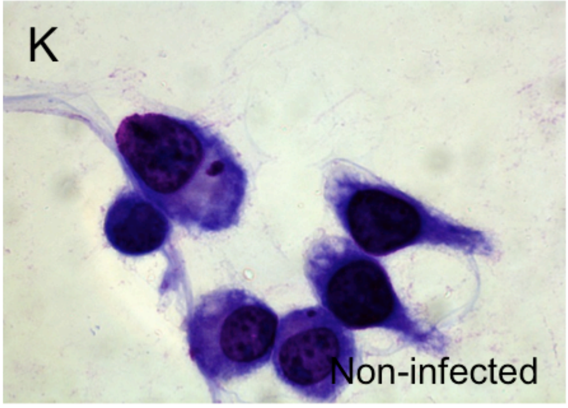


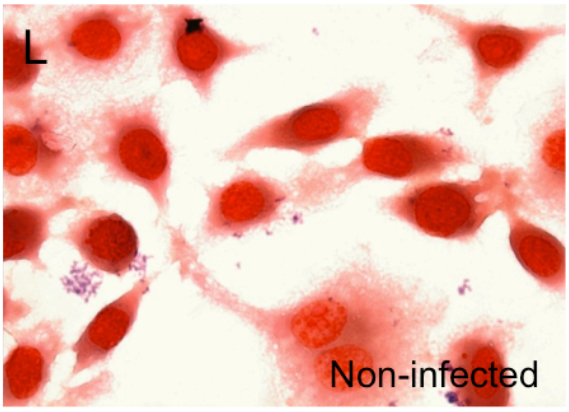


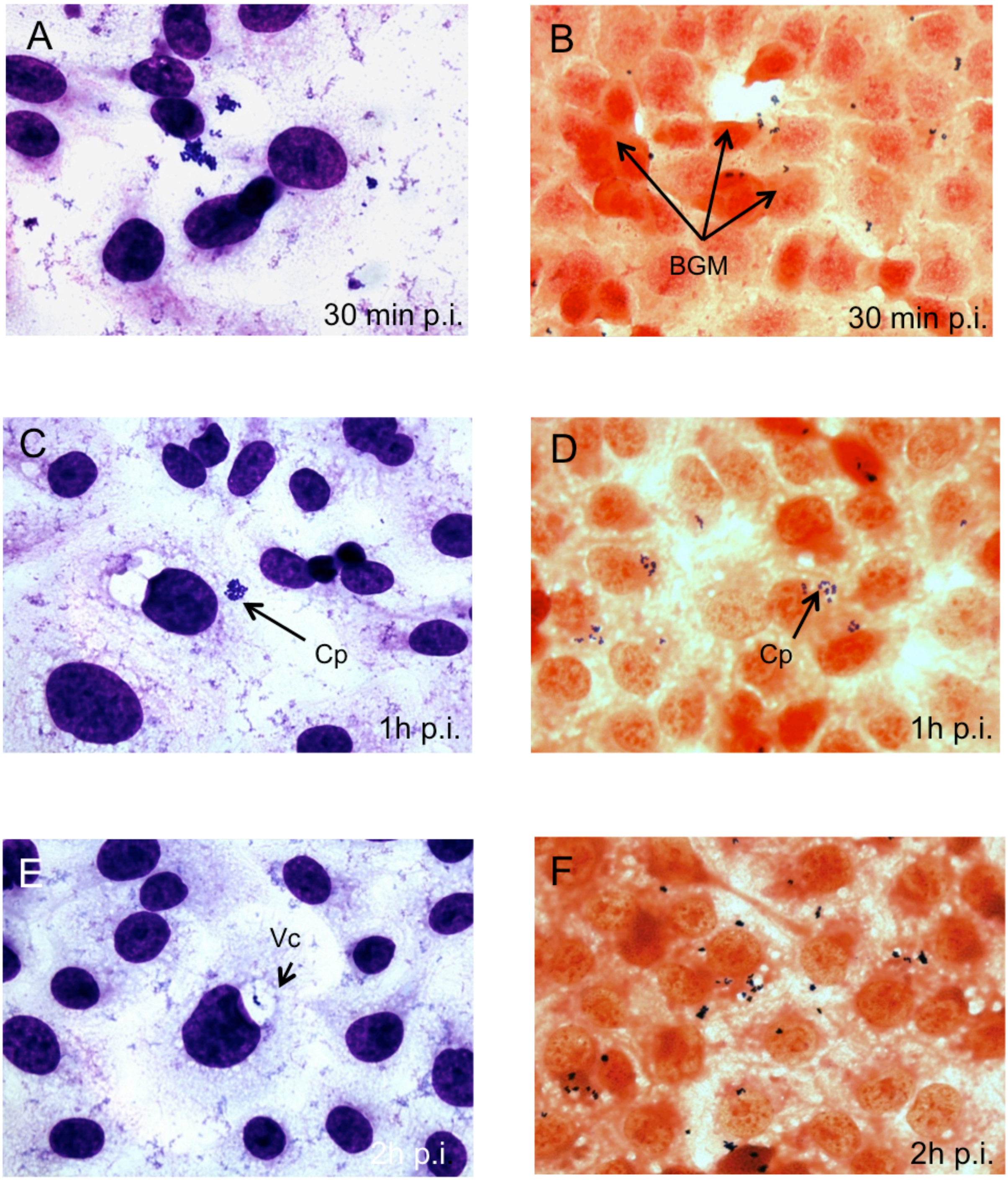
Fig. 8: Gentamicin Invasion Assay with macrophages (Mp) stained by May Gruenwald-Giemsa (A, C, E, G, I and K) and Gram (B, D, F, H, J and L)

A, B: 30 minutes post infection (p.i.), *C. pseudotuberculosis* (Cp) is already visible inside the cells as small clumps of blue-stained coccoid bacilli. C, D: After one hour of infection, the bacteria can be seen in large vacuoles (Vc) in the cytoplasm. E, F: At 2 hours p.i., the bacteria seem to have escaped from the vacuoles and are located in the cytoplasm. G, H: At 4 hours p.i., the bacteria are mainly found in the proximity of the cell nucleus. I, J: At 24 hours p.i., the bacteria have significantly multiplied inside the cells; large clumps of bacteria are visible next to the debris of their destroyed host cells. K, L: The non-infected negative controls show a normal morphology after 24 hours of incubation.

6.1.2. Microscopy of the BGM cells infected with *C. pseudotuberculosis*

BGM cells are an epithelial cell line originated from African green monkey kidney. They were chosen for the invasion assays in order to evaluate the capacity of *C. pseudotuberculosis* to invade non-phagocytic cells. On microscopic examination, the BGM cells showed an epitheloid growth pattern and formed a densely grown, confluent monolayer. The cells were fusiform with an abundant cytoplasm that appeared reddish-orange in Gram stains and bluish-purple when stained by May Gruenwald-Giemsa.

After 30 minutes of incubation with *C. pseudotuberculosis*, small clumps of bacteria were already discernible within the cytoplasm of the BGM cells. The number of internalized bacteria was significantly lower when compared to the macrophages. On average, the cells contained between 2 and 10 bacteria, which were mainly located in the proximity to the cell nucleus but could, occasionally, also be found within large cytoplasmatic vacuoles. At 4 hours post infection, the bacteria had started to multiply within the cells and a considerable increase in the number of intracellular bacteria was visible after 24 hours of infection. The monolayers appeared disrupted as cells that were strongly parasited by bacteria underwent degeneration and detached from the glass coverslips. Clumps of bacteria and debris of destroyed cells could be observed. Non-infected BGM cells showed a normal morphology and no signs of degeneration (see Fig. 9)



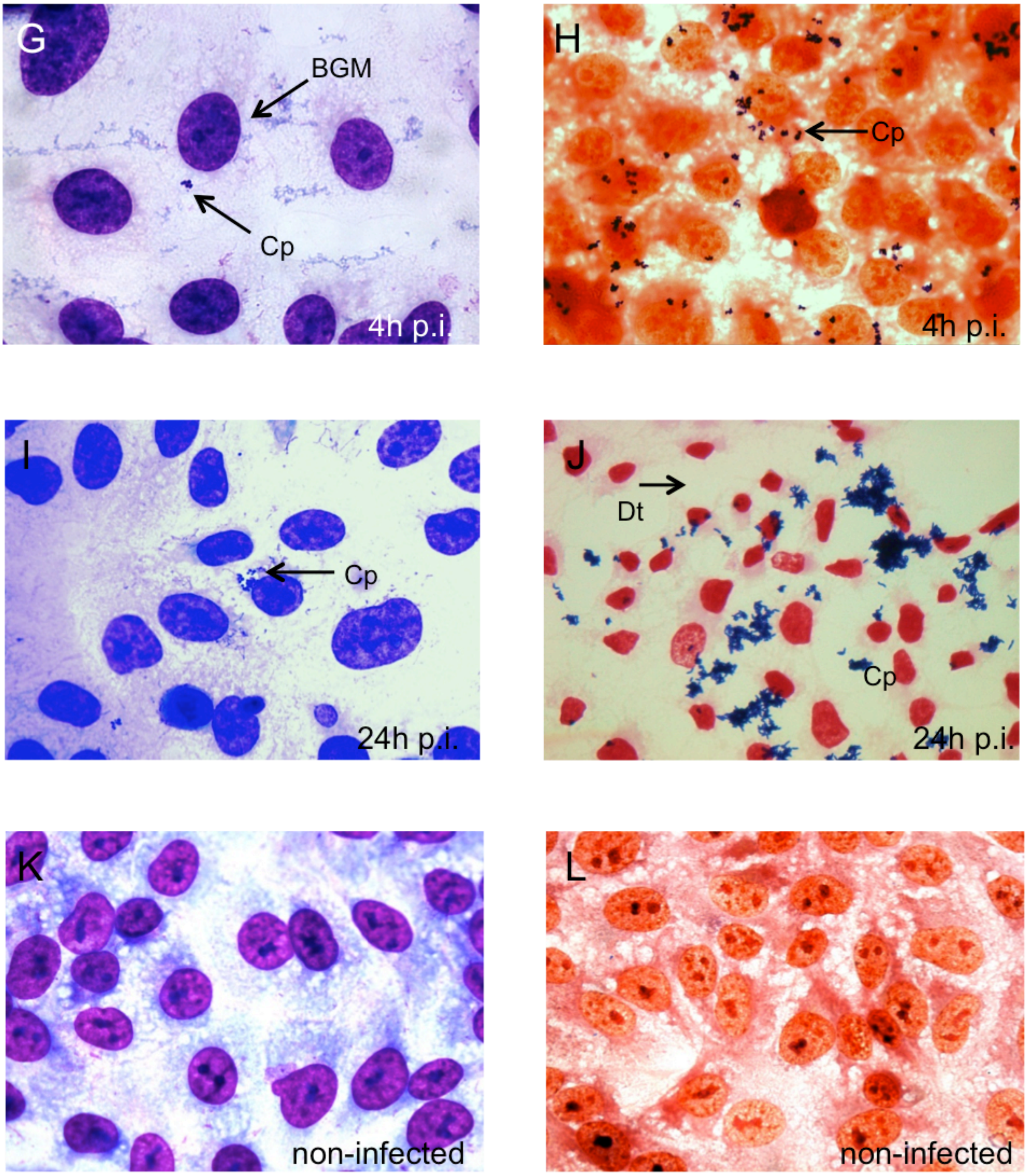


Fig. 9: Gentamicin assay with BGM cells (BGM), stained by May Gruenwald-Giemsa (A, C, E, G, I and K) and Gram (B, D, F, H, J and L)

At 30 minutes p.i., the corynebacteria (Cp) can be observed inside the BGM cells (A, B). On average, the cells contain 2-10 bacteria, which can be found near the cell nucleus and within large vacuoles (Vc) in the cytoplasm (C, D, E, and F). G, H: The bacteria have started to multiply within their host cells at 4 hours p.i.. I, J: At 24 p.i., the number of intracellular bacteria has significantly increased; cell debris (Dt) and clumps of extracellular bacteria can be seen. K, L: Undisrupted monolayers of non-infected BGM cells after 24 hours of incubation.

6.1.3. Microscopy of the macrophages and BGM cells infected with formalin-inactivated *C. pseudotuberculosis*

To investigate whether *C. pseudotuberculosis* actively induces its uptake into macrophages and BGM cells, a gentamicin invasion assay was performed with formalin-inactivated bacteria. As inactivation with formalin attenuates bacterial virulence factors, formalin-killed bacteria should be incapacitated from invading cells. Macrophages and BGM cells were grown on glass coverslips, infected with formalin-inactivated *C. pseudotuberculosis*, fixed with methanol and stained by May Gruenwald-Giemsa. The cells were examined by light microscopy and the invasive behaviour of the formalin-inactivated bacteria was compared to the invasive capacity of viable *C. pseudotuberculosis*.

The formalin-killed bacteria were readily taken up by the macrophages and the progress of the internalized bacteria was similar to viable *C. pseudotuberculosis*. Already 30 minutes post infection, large clumps of bacteria could be observed within the cytoplasm of the macrophages, and after further 90 minutes of incubation, the bacteria were localized in large vacuoles. At 24 hours post infection, the number of intracellular bacteria had significantly decreased and fewer macrophages contained bacteria, indicating a destruction of the formalin-inactivated *C. pseudotuberculosis* (see Fig. 10).

In the case of the BGM cells, no intracellular bacteria could be observed after infection with inactivated strains of *C. pseudotuberculosis*. As seen in Fig. 10, large numbers of inactivated bacteria could be localized outside the cells at 30 minutes post infection, but no internalized bacteria were found. After 2 hours of incubation, the number of extracellular bacteria had significantly decreased and practically no bacteria could be detected at 24 hours post infection. The formalin-inactivated corynebacteria seemed unable to invade the BGM cells.

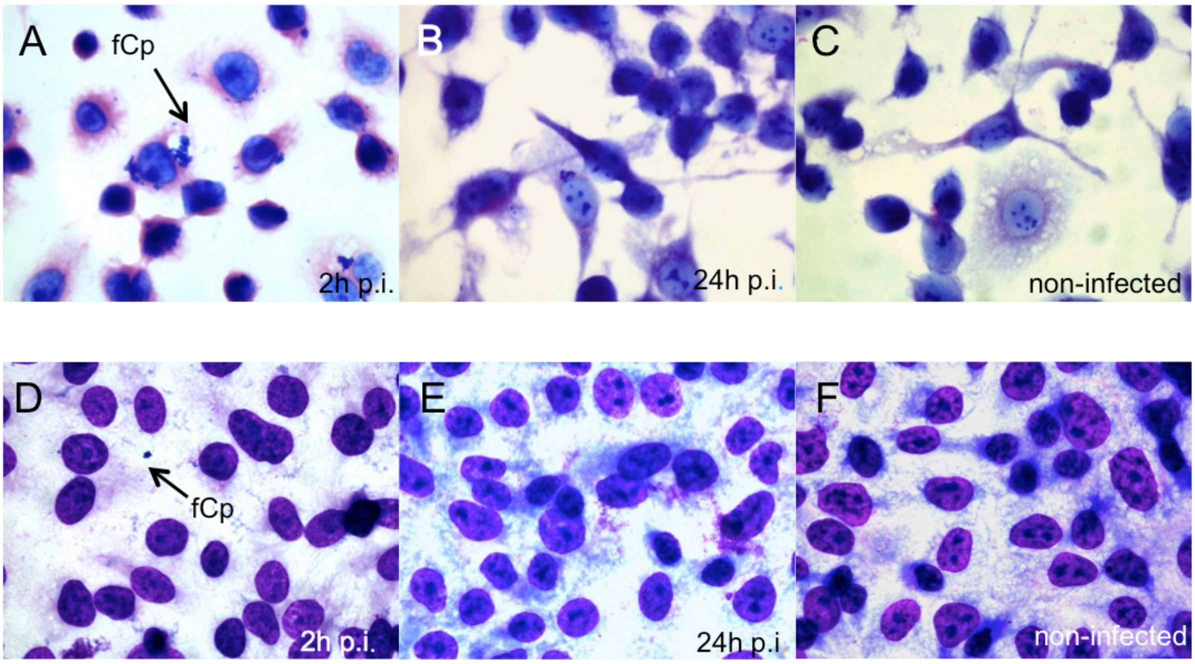


Fig. 10: Macrophages (A, B, and C) and BGM cells (D, E, and F) infected with formalin-inactivated *C. pseudotuberculosis* (fCp)

A: Large numbers of bacteria can be found within vacuoles and the cytoplasm of the macrophages at 2 hours p.i.. B: The number of internalized bacteria has significantly decreased at 24 hours p.i.. D, E: 2 hours p.i., only extracellular bacteria can be observed in the BGM cells and no bacteria can be detected at 24 hours post infection.

6.2. Scanning Electron Microscopy

To analyze the first step of interaction between the bacteria and host cells, the macrophages and BGM cells infected with *C. pseudotuberculosis* were examined at the ultrastructural level by SEM, in order to understand the mechanisms of attachment, engulfment and internalization of the bacteria. A gentamicin invasion assay was performed with both, macrophages and BGM cells, and samples were processed for SEM at different time points. The time course of the experiments was designed differently for macrophages and BGM cells since the kinetics of the internalization of *C. pseudotuberculosis* differed between the two cell types. The time points chosen were based on the findings of the gentamicin invasion assay, which showed that the internalization of the bacteria occurred very fast in the macrophages. Therefore, the macrophages were fixed at 5 minutes, 10 minutes, 20 minutes, 30 minutes and 60 minutes post infection while the BGM cells were fixed at 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours post infection.

The examination of the macrophages by SEM yielded no results since 5 minutes after the infection, the bacteria had mostly been taken up by the macrophages. The internalization of *C. pseudotuberculosis* proceeded so quickly that, even at the earliest time point of fixation, the bacteria could not be detected on the cell surface anymore.
The SEM analysis of the BGM cells showed that an intimate contact between the bacteria and the host cell membrane had already been established at 30 minutes post infection, but adherent bacteria could still be found on the cell surface 4 hours after the infection. *C. pseudotuberculosis* usually attached to the BGM cells in clumps of aggregated bacteria that formed grape-like structures on the cell surface. The adhesion of the bacteria could be observed best at 2 hours post infection; it was mediated by cellular protrusions that had formed on the cell surface at the site of bacterial attachment. Subsequently, these cellular protrusions appeared to elongate and extend to the entire length of the bacterial rod before they wrapped themselves around the bacteria (see Fig. 11). The engulfment was more frequently seen at 4 hours post infection; the interaction between *C. pseudotuberculosis* and the host cell protrusions seemed to induce an engagement of the local cell membrane, causing it to attach tightly to the adherent bacteria. Thus, the bacterial rods were sequentially engulfed by the cell membrane and taken up through dent-like structures on the cell surface (see Fig. 12).

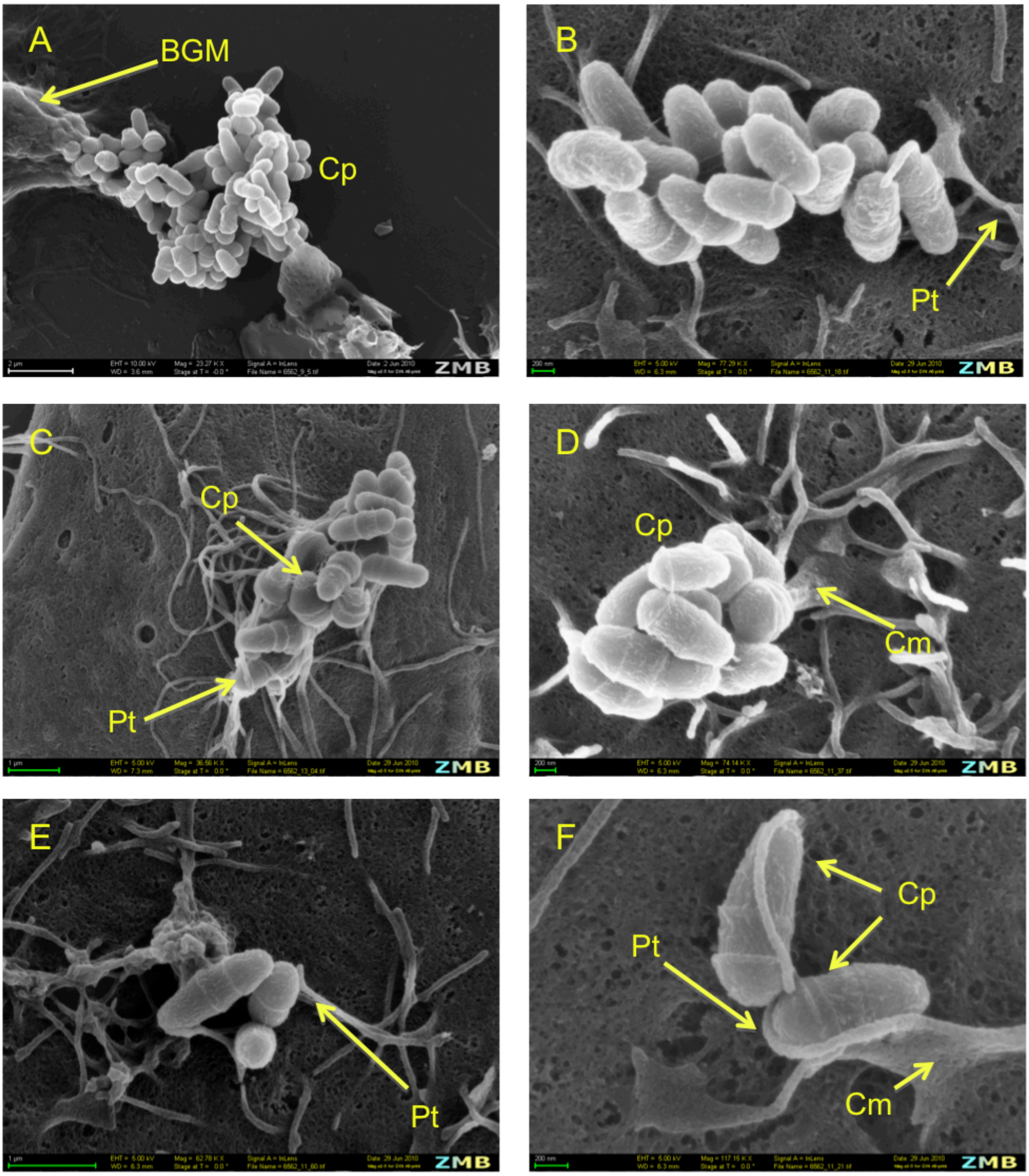


Fig. 11: SEM analysis of the BGM cells at 2 hours post infection

A, B: Clumps of aggregated bacteria (Cp) adhere to the cell membrane (Cm). C, D: The adhesion is mediated through cellular protrusions (Pt) that have formed at the site of bacterial attachment. E, F: The protrusions extend to the entire length of the rods and wrap themselves around the bacterial cells. F: The interaction between the cellular protrusions and the bacteria induce the engagement of the local cell membrane, which attaches tightly to the adherent bacteria.

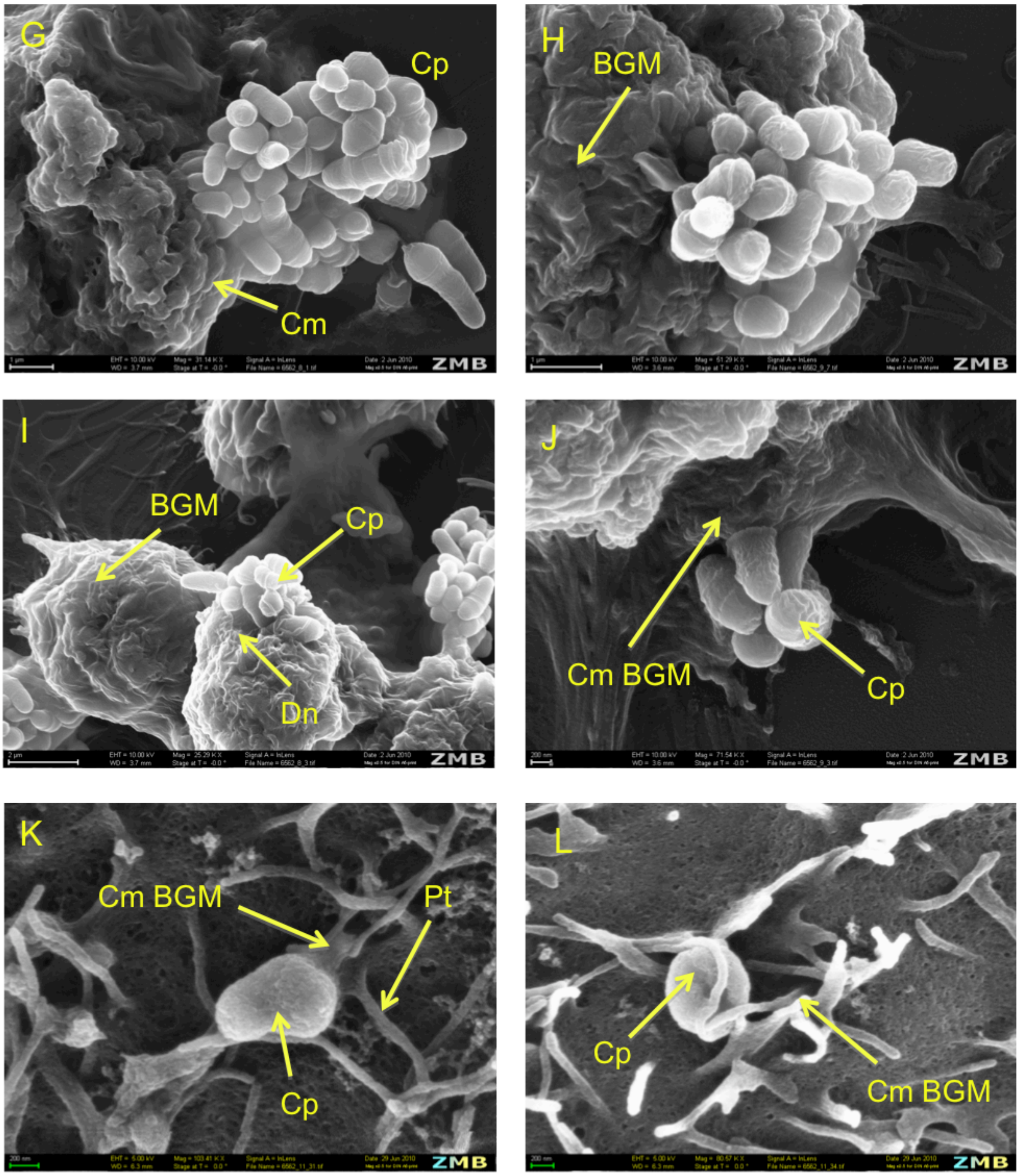


Fig. 12: SEM analysis oft the BGM cells at 4 hours post infection

G, H: The cell membrane (Cm BGM) wraps around the bacterial cells (Cp) and sequentially engulfs the rods. I, J: The bacteria are taken up through dent-like structures (Dn) in the cell membrane. K, L: Cellular protrusions wrapped around a single bacterial cell that is subsequently engulfed by the cell membrane and taken up through a dent-like structure.

6.3. Gentamicin Invasion Assay

6.3.1. Quantification of the internalization into macrophages and BGM cells

The gentamicin invasion assay was performed to assess the extent to which *C. pseudotuberculosis* enters macrophages and epithelial cells and to quantify the number of intracellular bacteria. At different time points (T_1 - T_5), the internalized bacteria were released through lysis of the cells and quantified by plate count method. The number of viable bacteria determined at a time point was expressed as the mean number +/- SD of CFU/ml and as the percentage of the inoculum added to the cell monolayers. The data were obtained from 5 different gentamicin invasion assays and constitute the average number of viable bacteria recovered on plates at this time point.

The first sample was taken at 30 minutes post infection (T_1), before fresh medium containing gentamicin was added to the monolayers. The number of viable bacteria recovered at this time point represented the cell-associated bacteria, i.e. the extra- and intracellular bacteria, and corresponded to the inoculum and the viable bacteria in the supernatant. The number of cell-associated bacteria was consistent in all experiments and similar in macrophages and BGM cells. After 30 minutes (T_2) and 90 minutes (T_3) of treatment with gentamicin, the average number of viable bacteria obtained from the plates decreased in both, macrophages and BGM cells, as only internalized bacteria were detected at these time points. The gentamicin was removed 2 hours after the infection (T_3), and at 4 hours post infection (T_4), a slight increase in the number of intracellular bacteria was visible. After 24 hours of incubation, the number of intracellular bacteria continued to be higher than at 2 hours post infection in both cell lines (see Fig. 13).

The results of the gentamicin invasion assay demonstrated that *C. pseudotuberculosis* is able to enter and multiply within macrophages and BGM cells, but the levels of internalization varied between the two cell lines. Despite the lower MOI used for the infection of the macrophages, the number of viable bacteria associated with the macrophages was found to be higher than in the BGM cells (see Table 1).

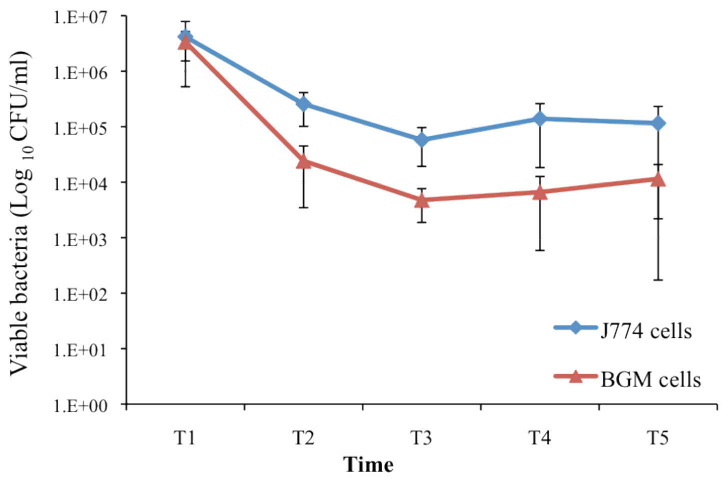


Fig. 13: Gentamicin Invasion Assay

Viable (Log_{10} CFU/ml +/- SD) *C. pseudotuberculosis* recovered from the macrophages and BGM cells at different times of infection. At 30 minutes post infection (T_1), the monolayers were washed and further incubated with fresh medium containing 100 µg/ml gentamicin for 90 minutes. Samples were taken at 30 minutes (T_1), 1 hour (T_2), 2 hours (T_3), 4 hours (T_4) and 24 hours (T_5) post infection. Data represent average of 5 different gentamicin assays.

Table 1: Adherence and internalization into J774 macrophages and BGM cells after infection with *C. pseudotuberculosis*

Cell line	Number of viable bacteria (CFU/ml)[a]	
	T_1 (30 minutes p.i.) Extra- and intracellular[b]	T_3 (2 hours p.i.) Intracellular[c]
Macrophages (MOI 10)	4.2×10^6 +/- 3.6×10^6	5.8×10^4 +/- 3.9×10^4
BGM-cells (MOI 100)	3.4×10^6 +/- 1.9×10^6	4.8×10^3 +/- 2.8×10^3

[a] Values represent the average number of viable bacteria recovered on agar plates at a time point expressed as CFU/ml (mean and standard deviation of 5 different experiments)
[b] Number of viable cell-associated bacteria (extra- and intracellular) recovered from the gentamicin-untreated monolayers at 30 minutes post infection.
[c] Number of viable intracellular bacteria recovered from the monolayers after treatment with gentamicin for 90 minutes at 2 hours post infection.

Based on the findings of the gentamicin invasion assay, the internalization efficiency of the two cell lines was compared by expressing the number of viable bacteria recovered from the monolayers as a percentage of the inoculum added to the cells. The results shown in Table 2 indicated that the level of adherence and internalization was significantly higher in macrophages than in BGM cells. While 50.25% of the inoculum could be recovered from the macrophage monolayers at 30 minutes post infection, the viable bacteria obtained from the BGM cells constituted only 0.34% of the inoculum.

Table 2: Number of viable cell-associated (extra- and intracellular) and internalized (intracellular) bacteria recovered from the monolayers expressed as the percentage of the inoculum added to the cells

Cell line	Number of viable bacteria (%)[a]		% Internalization[b]
	T_1 (30 minutes p.i.) Extra- and intracellular	T_3 (2 hours p.i.) Intracellular	
Inoculum macrophages 8×10^6 CFU/ml (MOI 10)	52.25 %	0.72 %	1.3 %
Inoculum BGM cells 1×10^9 CFU/ml (MOI 100)	0.34 %	0.00047%	0.1%

[a] Values represent the average number of viable bacteria recovered at 30 minutes and at 2 hours post infection expressed as the percentage of the inoculum added to the cells (mean of 5 different experiments)
[b] Ratio of number of internalized bacteria (average number of viable bacteria recovered at 2 hours post infection) to number of cell-associated bacteria (average number of bacteria recovered at 30 minutes post infection) .

Furthermore, when the ratios of the percentages of intracellular bacteria (T_3) to cell-associated bacteria (T_1) were compared for the two cell lines, the macrophages showed an internalization rate that exceeded the level of internalization in the BGM cells by a factor of ten; 1.3% of cell-associated *C. pseudotuberculosis* invaded the macrophages while only 0.1% of cell-associated bacteria were able to invade the BGM cells (see Fig. 14).

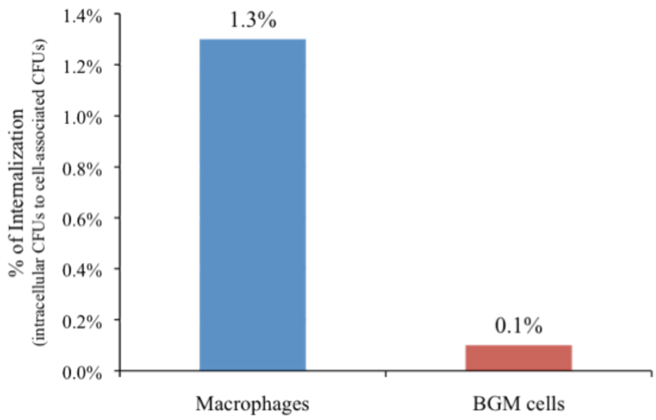


Fig. 14: Comparison of the level of internalization of *C. pseudotuberculosis* by macrophages and BGM cells
The percentage of internalization was expressed as the ratio of intracellular bacteria recovered at 2 hours post infection to cell-associated bacteria recovered from the gentamicin-untreated monolayers at 30 minutes p.i.. The internalization rate of the macrophages exceeded the internalization rates of the BGM cells by a factor of ten.

6.3.2. Inhibition of *C. pseudotuberculosis* invasion

To investigate the host signalling molecules and cytoskeletal components involved in bacterial entry into macrophages and BGM cells, the effects of specific inhibitors on the internalization frequency of *C. pseudotuberculosis* were examined. The macrophages and BGM cells were pretreated with 7 different inhibitors of distinct eukaryotic cellular functions at varying concentrations for 30 minutes before infection with *C. pseudotuberculosis*. A gentamicin invasion assay was performed with the treated cells and, simultaneously, with untreated cells that served as a negative control. To assess the internalization frequency, the mean number of viable intracellular bacteria (CFU/ml +/- SD) recovered on agar plates after cell lysis at two hours post infection was determined and compared between treated and untreated cells. The data were expressed as CFU/ml and as a percentage of the negative control. The inhibitors did not exert a significant effect on the cellular or bacterial viability.

6.3.2.1. Cytochalasin D and Colchicine

The macrophages and BGM cells were treated with the inhibitors cytochalasin D and colchicine to invesigate the involvement of the host cytoskeleton in the uptake of *C. pseudotuberculosis*. Cytochalasin D blocks the F-actin polymerization and inhibits the formation of the eukaryotic microfilaments (Bertuccini et al., 2004). The actin cytoskeleton participates in many important cellular processes, including cell motility, cell division, vesicle and organelle movement, cell signalling, and the establishment of cell junctions. The microfilaments also play an important role during phagocytosis providing the cell membrane movement necessary for the engulfment of a particle. Phagocytosis in macrophages and the uptake of various bacterial pathogens into their host cells are known to be dependent on the actin cytoskeleton and can be inhibited by cytochalasin D (Dramsi and Cossart, 1998). Although most bacterial pathogens exploit the actin cytoskeleton for their internalization into cells, some invasive pathogens appear to be dependent on microtubules for a successful invasion into their host cells. It was shown that the microtubule depolymerizing agent colchicine was able to inhibit the entry of these pathogens. The microtubules constitute a major component of the cytoskeleton in eukaryotic cells and play an essential role in chromosome separation during mitosis, organelle movement, signal transduction and modulation of the actin dynamics. (Yoshida et al., 2003).

Cytochalasin D was added to the cells at three different concentrations (10 μM, 50 μM and 100 μM) while colchicine was used at a concentration of 10 μg/ml. Cytochalasin D showed a strong inhibitory effect on the internalization rates of *C. pseudotuberculosis* in both, macrophages and BGM cells. In the BGM cells, the decrease of viable intracellular bacteria was more evident at the highest dose utilized, leading to a reduction of the internalization levels of 97%, while the lower doses decreased the percentages of the number of internalized bacteria to 28% of those of the untreated cells. In the macrophages, the inhibitory effect of cytochalasin D was even more pronounced; no viable bacteria could be detected on agar plates after cell lysis at two hours post infection. All three doses of cytochalasin used decreased the number of intracellular bacteria to less than 0.005% of those recovered from the untreated cells. Treatment of the macrophages with cytochalasin D seemed to inhibit the uptake of *C. pseudotuberculosis* up to nearly 100%, with no dose-dependent effect being distinguishable (see Fig. 15 and Fig. 16).

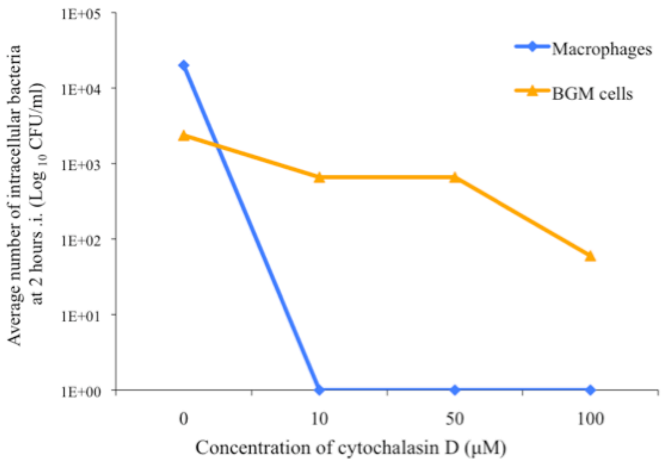


Fig: 15: Effect of cytochalasin D on *C. pseudotuberculosis* internalization into macrophages and BGM cells.

The viable intracellular bacteria were determined at 2 hours post infection as Log_{10} CFU/ml recovered from the agar plates after cell lysis. *C. pseudotuberculosis* entry into macrophages was strongly affected by cytochalasin D; at 2 hours p.i. no viable intracellular bacteria could be detected after treatment with different doses of cytochalasin D. In the BGM cells, the internalization rates decreased in a dose-dependent manner after treatment with cytochalasin D; the inhibitory effect was more pronounced at higher doses.

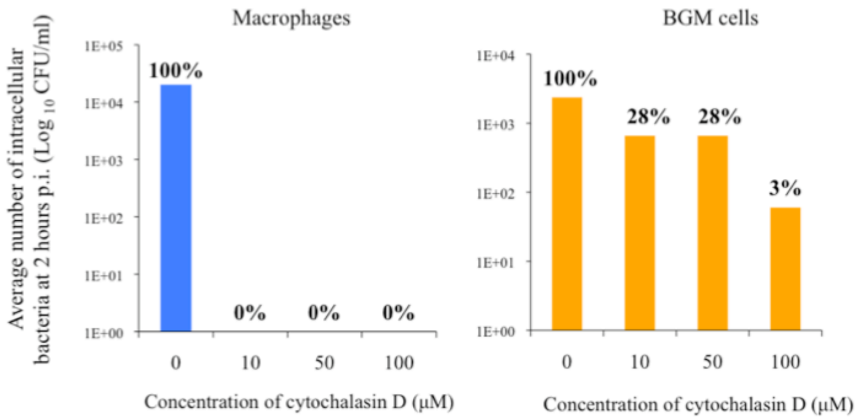


Fig. 16: Viable intracellular bacteria recovered at 2 hours post infection expressed as the percentage of the intracellular bacteria recovered from the untreated cells.

Cytochalasin D lead to a reduction of *C. pseudotuberculosis* entry into macrophages of nearly 100%. In the BGM cells, the percentage of viable intracellular bacteria was reduced to 28% and 3%, respectively, of the negative control after treatment with increasing doses of cytochalasin D.

Treatment of the cells with 10 µg/ml colchicine had no measurable effect on the viable bacteria recovered on agar plates in the gentamicin invasion assay, indicating that microtubules did not seem to be involved in *C. pseudotuberculosis* entry into macrophages and BGM cells.

6.3.2.2. Genistein

Genistein, a broad-spectrum TPK-inhibitor (Hirata et al., 2002; Kwok et al., 2002), was used to analyze whether the invasion process was dependent on the host phosphotyrosine signalling cascade. TPKs play an important role in FcγR-mediated phagocytosis transmitting the phagocytic signal from the membrane receptors to the actin cytoskeleton (Kwiatkowska and Sobota, 1999; Aderem and Underhill; 1999). Genistein was added to the cells at a concentration of 100 µM and 400 µM.

As seen in Fig. 17, the treatment with genistein resulted in a decrease of *C. pseudotuberculosis* entry in both, macrophages and BGM cells. The effect was more pronounced at higher doses and the decrease in the internalization levels was slightly more distinct in the macrophages. The percentage of viable bacteria recovered from macrophages treated with 100 µM genistein was reduced to 35% of those recovered in untreated cells and to 0.1% when treated with 400 µM of genistein. In the BGM cells, the number of viable intracellular bacteria was reduced to 44% and 6%, respectively, of the internalized bacteria in the untreated cells (see Fig. 18).

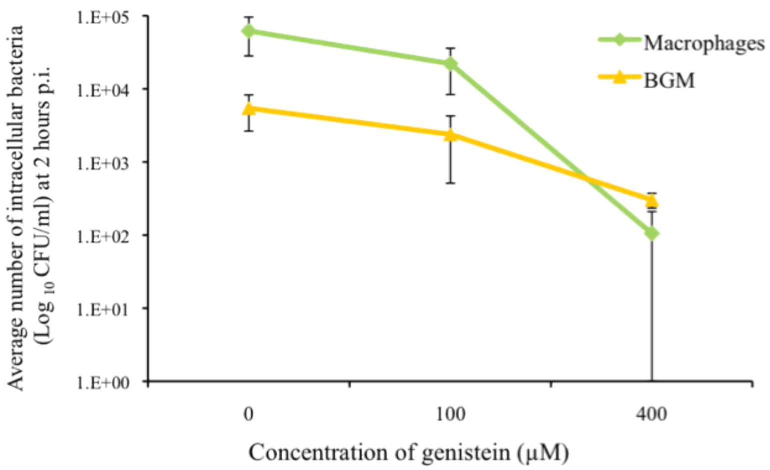


Fig. 17: Effect of genistein on the internalization levels of *C. pseudotuberculosis* in macrophages and BGM cells.
The mean number of viable intracellular bacteria (CFU/ml) +/- SD recovered at 2 hours post infection was compared between treated and untreated cells. The treatment with genistein lead to a decrease in internalization levels in macrophages and BGM cells. The effect was more pronounced at a dose of 400 µM genistein.

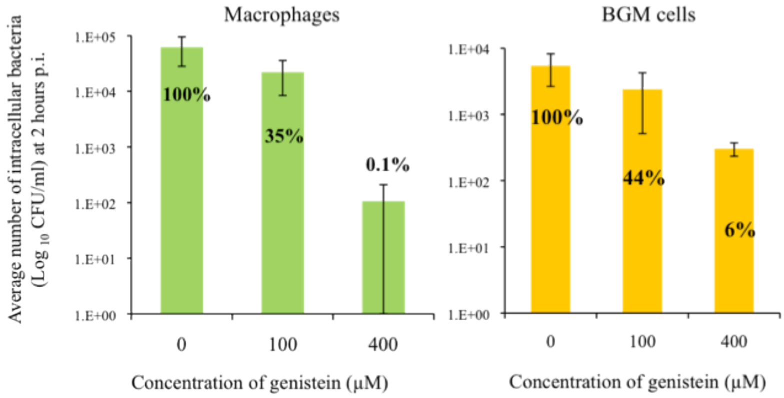


Fig. 18: Viable intracellular bacteria recovered at 2 hours post infection expressed as the percentage of the intracellular bacteria recovered from the untreated cells.
Treatment with 100 µM genistein reduced the percentage of viable bacteria to 35% in macrophages and to 44% in BGM cells of those recovered in non-treated cells. In cells treated with 400 µM genistein, the inhibitory effect was even more pronounced; the internalization was reduced by over 90%.

To compare the percentages of internalization between treated and untreated cells, the number of viable bacteria recovered at 2 hours post infection was expressed as the ratio of the percentages of internalized bacteria to cell-associated bacteria and as a percentage of the inoculum. Table 3 shows that the percentage of internalization was significantly lower in the cells treated with 400 µM of genistein.

Table 3: Average number of viable bacteria recovered at 2 hours p.i. expressed as a percentage of the inoculum and as a percentage of internalization

Cell line	Number of viable intracellular bacteria at 2 hours p.i. %		
	0µM Genistein	100µM Genistein	400µM Genistein
Macrophages			
% of Inoculum[a]	0.7%	0.2%	0.001%
% Internalization[c]	7%	7%	0.02%
BGM cells			
% of Inoculum[b]	0.0005%	0.0002%	0.00003%
% Internalization[c]	0.1%	0.07%	0.001%

[a] Macrophages were infected with 8 x 10^6 CFU/ml
[b] BGM cells were infected with 1 x 10^9 CFU/ml
[c] The percentage of internalization was calculated as the ratio of the mean number of internalized bacteria at 2 hours post infection to the mean number of cell-associated bacteria at 30 minutes p.i.

6.3.2.3. Wortmannin

To evaluate the involvement of the PI 3-kinase in the internalization process, increasing doses of wortmannin, a potent specific inhibitor of PI 3-kinase (Kwok et al., 2002; Bertuccini et al., 2004), were applied to the macrophages and BGM cells. PI 3-kinase is known to participate in the signalling events involved in FcγR-mediated phagocytosis and invasion of various bacterial pathogens into their host cells (Dramsi and Cossart, 1998; Aderem and Underhill; 1999; Cossart and Sansonetti, 2004).

Wortmannin inhibited the bacterial uptake into both, macrophages and BGM cells, in a dose-dependent manner. The effect of the inhibitor was similar in macrophages and BGM cells as to increasing doses of wortmannin lead to gradient decrease in the levels of internalized *C. pseudotuberculosis*. The decrease in the internalization levels was more dramatic when doses between 2 μM and 4 μM were used, reducing the percentage of the number of viable intracellular bacteria in the treated cells to less than 10% of those of the untreated cells. When higher doses were used, the effect levelled out with the number of internalized *C. pseudotuberculosis* decreasing from 0.6% to 0.3% in macrophages and from 0.4% to 0.2% in BGM cells of those of the untreated cells (see Fig. 19 and Fig. 20).

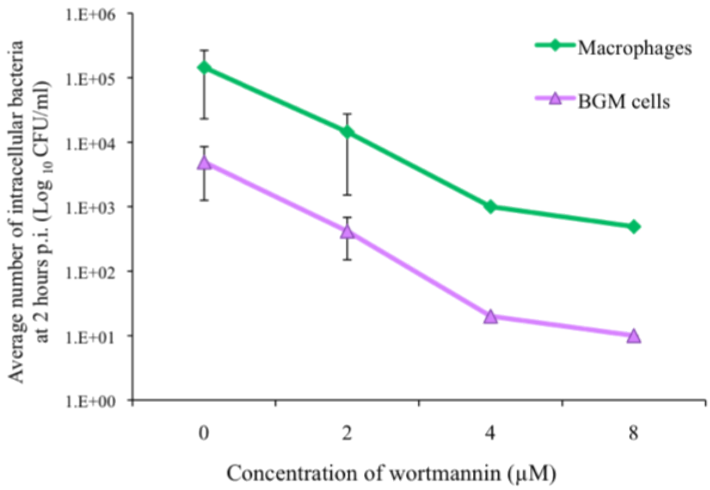


Fig. 19: Effect of wortmannin on the internalization of *C. pseudotuberculosis* into macrophages and BGM cells.
Treatment with increasing doses of wortmannin resulted in a gradient decrease in the number of viable intracellular bacteria (mean number of CFUs +/- SD at 2 hours p.i.) recovered from the treated cells when compared to the untreated negative controls. The effect was more pronounced at doses between 2 μM and 4 μM.

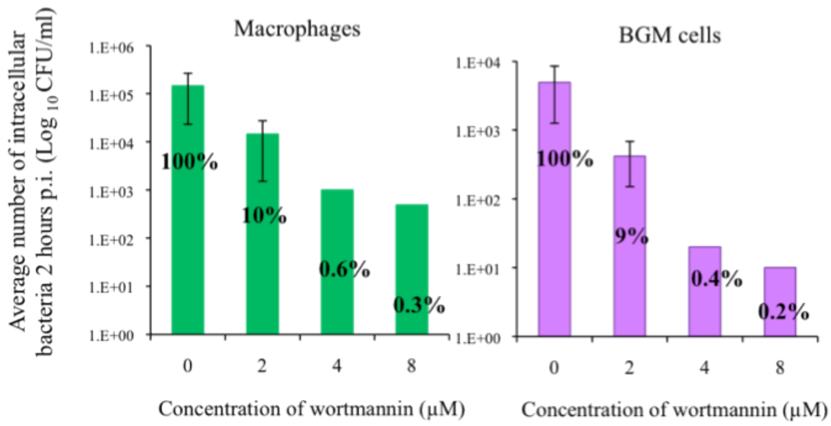


Fig. 20: Viable intracellular bacteria recovered at 2 hours p.i. expressed as the percentage of the intracellular bacteria recovered from the untreated cells. The percentages of the number of intracellular *C. pseudotuberculosis* decreased in macrophages and BGM cells to 10% and 9%, respectively, of those of the untreated cells after the treatment with 2 µM wortmannin. Treatment with 4 µM and 8 µM of wortmannin reduced the percentages of internalized bacteria to under 1% of those recovered in non-treated cells.

When the number of viable intracellular bacteria recovered from the cells at 2 hours post infection was expressed as a percentage of the inoculum and as a percentage of internalization (ratio of internalized CFU/ml to cell-associated CFU/ml) and compared between the treated and untreated cells, the percentages of internalization were found to decrease in a dose-dependent manner in the cells treated with wortmannin (see Table 4).

Table 4: Average number of viable bacteria recovered at 2 hours p.i. expressed as a percentage of the inoculum and as a percentage of internalization

Cell line	Number of viable intracellular bacteria at 2 hours p. i. %			
	0µM	2µM	4µM	8µM
Macrophages				
% Inoculum	1.8%	0.1%	0.01%	0.006%
% Internalization	18%	2%	0.4%	0.2%
BGM cells				
% Inoculum	0.0004%	0.00004%	0.000002%	0.000001%
% Internalization	0.1%	0.01%	0.005%	0.001%

[a] Macrophages were infected with 8 x 10^6 CFU/ml
[b] BGM cells were infected with 1 x 10^9 CFU/ml
[c] The percentage of internalization was calculated as the ratio of the mean number of internalized bacteria at 2 hours p.i. to the mean number of cell-associated bacteria at 30 minutes p.i.

6.3.2.4. Staurosporin

To examine whether the binding of *C. pseudotuberculosis* to the cell membrane evoked a signalling cascade mediated by PKCs, the macrophages and BGM cells were treated with staurosporin, a potent inhibitor of different protein kinases, including PKC, some phosphotyrosine kinases and cAMP-dependent protein kinases (Bertuccini et al., 2004). PKCs are involved in various signalling pathways in eukaryotic cells and also participate in signal transduction during phagocytosis (Kwiatkowska ad Sobota, 1999).

The cell monolayers were treated with increasing doses of staurosporin at a concentration of 0.5 µM, 2 µM, 4 µM and 6 µM. The treatment of the macrophages with 2 µM and 0.5 µM of staurosporin produced a considerable enhancement of the bacterial internalization into the cells. This effect was more relevant in the nanomolar range and higher doses of staurosporin did not influence the internalization rates as effectively as low doses. In the macrophages treated with 2 µM and 0.5 µM staurosporin (see Fig. 21), the number of internalized bacteria was 3 to 4 times higher that in the non-treated cells. In contrast, treatment of the BGM cells with increasing doses of staurosporin did not significantly affect the internalization rates of *C. pseudotuberculosis*.

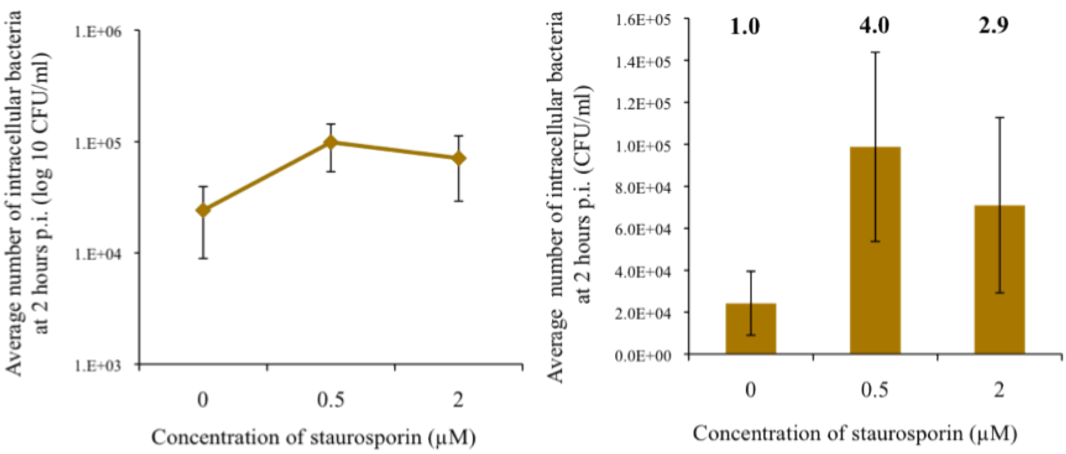


Fig. 21: Internalization rates of *C. pseudotuberculosis* in macrophages treated with staurosporin.
Treatment with low doses of staurosporin lead to a considerable enhancement of bacterial internalization; the cells treated with 0.5 µM staurosporin showed a 4-fold higher internalization rate while treatment with 2 µM staurosporin produced a 3-fold increase in the number of intracellular bacteria.

6.3.2.5. Sodium *ortho*-vanadate and Monodansylcadaverin

The macrophages and BGM cells were also treated with sodium *ortho*-vanadate, a component that inhibits the tyrosine phosphatases and ATPases (Kwok et al., 2002; Bertuccini et al., 2004), to further investigate the role of the tyrosine phosphorylation-related signalling pathway in the uptake of *C. pseudotuberculosis*. Moreover, monodansylcadaverine, a primary amine that blocks receptor recycling to the cellular membrane (Bertuccini et al., 2004), was used to evaluate the involvement of membrane receptors in the internalization process. Both inhibitors had no measurable effect on the internalization rates of *C. pseudotuberculosis*.

7. Discussion

The ability of *C. pseudotuberculosis* to persist as a facultative intracellular parasite plays an important role in the pathogenesis of CLA. In order to assess the impact of intracellular survival on the pathogenesis, epidemiology and control of the disease, the cell tropism and cell invasion mechanisms of the bacterium were investigated. Further, the bacteria-host interactions and the in-vitro ability of *C. pseudotuberculosis* to invade macrophages and epithelial cells were analyzed by means of a gentamicin invasion assay, bright field light microscopy and scanning electron microscopy.

7.1. Bright Field Light Microscopy of macrophages and BGM cells infected with *C. pseudotuberculosis*

The evaluation of the microscopic images confirmed the findings of other authors (Hard, 1972; Tashjian and Campbell, 1982) that *C. pseudotuberculosis* is able to survive and multiply within macrophages. In addition, the presented microscopic studies revealed that *C. pseudotuberculosis* was localized inside epithelial BGM cells and this study was, therefore, able to demonstrate for the first time that the bacterium is also capable of invading and multiplying within non-phagocytic cells. The bacteria were considered to be intracellular because of the gentamicin selection performed in this invasion assay.

In both, macrophages and BGM cells, *C. pseudotuberculosis* was able to multiply within the cytoplasm, and cells that were heavily parasited by bacteria underwent degeneration while the bacteria survived. This observation is consistent with the findings of Hard and Tashjian et al., (1972; 1982) who showed that *C. pseudotuberculosis* survives in macrophages and causes the death of its host cells. Tashjian et al. and Hard further suggested that the layer of mycolic acids on the cell surface allows the bacterium to survive the degradative mechanisms of the macrophages and causes the degeneration of the host cells due to its cytotoxic properties.
In this study, the corynebacteria were often found in large cytoplasmatic vacuoles within the macrophages at the early stages of the infection. Based on the electron microscopy studies performed by Hard (1972), these vacuoles were interpreted as phagosomes or

phagolysosomes. In the later stages of the infection, *C. pseudotuberculosis* was localized mainly in the cytoplasm in proximity to the cell nucleus, suggesting that the bacterium is not only able to survive within phagosomes but possesses mechanisms to actively escape from the phagosomes. This hypothesis is supported by the results of a confocal laser scanning microscopy (CLSM) study that was performed in this institute in 2008. In this study, macrophages infected with *C. pseudotuberculosis* were stained by FITC, DAPI and FM4-64 to visualize the bacteria, cell nucleus and eukaryotic cell membranes. They found that *C. pseudotuberculosis* was localized in membrane-coated vacuoles inside the cytoplasm shortly after the infection. 24 hours post infection though, the bacteria could be detected free in the cytoplasm without surrounding membrane, indicating that the corynebacteria had escaped from the phagosome. (Niederer, 2008). A similar strategy to evade phagosomal degradation has been described for *L. monocytogenes*, an invasive pathogen that can multiply within a variety of cells, including epithelial cells and J774 mouse macrophages. Even though the fusion between phagosome and lysosome occurs soon after the uptake of the bacteria, *L. monocytogenes* is able to disrupt the phagosome membrane and evade into the cytoplasm by producing listeriolysin O and an extracellular phosphoryl-choline phospholipase C (Chastellier et al., 1994). Indeed, it has been suggested that the PLD produced by *C. pseudotuberculosis* might also play a role in the escape from the phagosome and macrophage death (McKean et al., 2007).

To investigate whether *C. pseudotuberculosis* actively induces its uptake into the macrophages and BGM cells, gentamicin invasion assays were performed with formalin-inactivated bacteria. BGM cells are an epithelial cell line and, as such, possess only a limited phagocytic capacity. The presented results confirmed the hypothesis that *C. pseudotuberculosis* employs an active mechanism to induce its own uptake into epithelial cells seeing that the inactivated bacteria were not able to invade the BGM cells. Andrade et al. (1989) have suggested that the invasion into non-phagocytic cells requires viable, metabolically active bacteria since the UV-inactivation of enteropathogenic *E. coli* prevented their invasion into HEp-2 cells.

In contrast, the formalin-inactivated bacteria were readily taken up by the macrophages and could also be found in phagosomes. However, the formalin-inactivated bacteria were not able to multiply and, after 24 hours of incubation, fewer intracellular bacteria were observed, indicating that the corynebacteria were digested in the phagolysosomes. These findings correspond with the observation that viable *Legionella pneumophila* are able to prevent the fusion of their phagosome with lysosomes, and thus evade degradation, while the formalin-killed bacteria are rapidly digested (Horwitz, 1983). As only live bacteria were able to multiply within the macrophages, it appears that *C. pseudotuberculosis* may employ active mechanisms to escape from the phagosomes and survive within phagocytic cells.

The formalin-inactivated bacteria are most likely taken up by the macrophages through phagocytosis, but it remains unclear whether the internalization of viable *C. pseudotuberculosis* into macrophages occurs primarly via phagocytosis or if the bacterium actively induces its uptake into phagocytic cells. Bacteria parasitizing phagocytic cells can either utilize pre-existing phagocytic mechanisms for their internalization or employ active invasion mechanisms to mediate entry into the cells. *Y. pseudotuberculosis*, for instance, binds preferentially to integrin receptors of phagocytic cells rather than binding to the normal receptors of phagocytosis. It is possible that, by utilizing active invasion pathways, these bacteria also bypass the traditional phagosome target pathways and thereby avoid normal degradation (Finlay and Falkow, 1997).

7.2. Scanning Electron Microscopy

The SEM studies of the macrophages revealed that the uptake of *C. pseudotuberculosis* was a rapid process: the first step of interaction between the macrophages and *C. pseudotuberculosis* could not be observed since most of the bacteria had already been taken up at 5 minutes post infection. A similar internalization rate has been described for group B streptococci in an invasion assay with J774 mouse macrophages; in this study, almost 50% of the inoculum could be recovered from the antibiotic-treated macrophages after an invasion period of 5 minutes (Valentin-Weigand et al., 1996).

The examination of the BGM cells showed that *C. pseudotuberculosis* adheres to the cell surface in clusters. The tendency to form clumps, especially when suspended in liquid media, is a well-known feature of the bacterium, which has been attributed to the hydrophobic nature of its outer layer (Dorella et al., 2005). Clumping of bacteria has also been considered a virulence factor as it reduces their susceptibility to host defence mechanisms. The fibronectin binding proteins of *Staphylococcus aureus*, for instance, mediate clumping of the bacteria by binding to host fibronectin and thereby enhance the bacterial adhesion and internalization into bovine mammary cells (Lammers et al., 1999). Furthermore, Hirata et al. (2004) observed that *C. diphteriae* exhibits two patterns of adherence: a localized and a diffuse pattern of adherence. Bertuccini et al. (2004) showed later that *C. diphteriae* strain 3319 colonized epithelial cells in small clusters, confirming their light microscopy observations that the bacterium associated with the epithelium in a localized adherence pattern. A localized pattern of adherence has also been described for enteropathogenic *E. coli*, which attach to the cell surface in discrete clusters (Andrade et al., 1989). The results of the presented SEM studies suggest that *C. pseudotuberculosis* might also exhibit a specialized pattern of adherence similar to *C. diphteriae* or *E. coli*.

The adherence of *C. pseudotuberuculosis* to the BGM cells induced cellular membrane protrusions at the site of attachment, which seemed to establish an intimate contact with the bacteria. Consecutively, these membrane pseudopods extended towards the entire length of the bacterium and lead to a sequential engulfment of the bacilli. Thereby, the cell surface showed no dramatic morphological changes apart from the local invaginations that appeared to have “zipped” along the bacterial rods and mediated the uptake through dip-like structures in the cell membrane. These SEM observations indicate that the bacterial attachment had a direct but extremely localized effect on the membrane structure, inducing the formation of the membrane pseudopods and subsequent engulfment. This uptake process is reminiscent of the “zipper-like” mechanism of phagocytosis involved in the invasion of *L. monocytogenes*, *Helicobacter pylori* (*H. pylori*) or *C. diptheriae*. The “zipper-like” mechanism is characterized by invagination of the host cell membrane at the site of the bacterial binding to the extent that the membrane “zips” up around the entire surface of the bacterium. The membrane protrusions are thereby mediated by cytoskeletal rearrangements in the area of bacterial entry, a process usually initiated by host signalling molecules (Mengaud, 1996; Kwok, 2002; Bertuccini et al., 2004). In contrast, the uptake of *S. typhimurium* or *S. flexneri* occurs via a process similar to macropinoyctosis, characterized by dramatic morphological changes of the membrane known as ruffles (Mengaud et al., 1996; Kwok et al., 2002). No such structures could be observed in the SEM pictures of the BGM cells, suggesting that *C. pseudotuberculosis* invades the BGM cells via a “zipper-like” mechanism of endocytosis.

Analogous to *L. monocytogenes* or *H. pylori*, the uptake of *C. pseudotuberculosis* may be induced by the interaction with membrane receptors and involve host signalling events and the rearrangement of the local host cytoskeleton.

7.3. Gentamicin Invasion Assay and inhibition of *C. pseudotuberculosis* invasion

The quantification of the viable intracellular bacteria recovered from the macrophages and BGM cells supported the results of the light microscopy observations showing that *C. pseudotuberculosis* is able to invade, survive and multiply within macrophages and epithelial cells.

The internalization rates were significantly different for the two cell lines: despite infecting the BGM cells with a 10-fold higher MOI, the macrophages showed an internalization rate of 1.3% while the BGM cells were invaded at rate of 0.1%. Cell-dependent invasion rates have also been reported for other invasive pathogens. *H. pylory*, for instance, was shown to invade gastric adenocarcinoma cells at a rate of 2.5% while hepatocytes were invaded at a rate of nearly 20% (Ito et al., 2008). Bertuccini et al. (2004) observed that the internalization rates of *C. diphteriae* were significantly higher in D562 cells than in HEp-2 cells. Both groups also noted that the entry efficiency was strongly dependent on the bacterial strain used in the invasion experiments. Comparing the number of viable intracellular *C. pseudotuberculosis* recovered from the BGM cells with other invasive pathogens, it appears that the bacterium enters the BGM cells at a lower level than *H. pylori*, *S. typhimurium* or *Y. pseudotuberculosis* but at a higher level than *Neisseria gonorrhoea*. The extent of entry into the macrophages was within the same range as for the other described pathogens (Kwok et al. 2002). However, it cannot be excluded that the efficient internalization rates observed in the macrophages were caused by phagocytosis rather than active invasion. The internalized bacteria recovered from the BGM cells could not result from phagocytosis as epithelial cells are not professional phagocytes. Overall, these findings indicate that *C. pseudotuberculosis* is capable of invading non-phagocytic cells at a moderate but slightly lower level than other pathogens.

With regards to the exact calculation of internalized bacteria, certain restrictions of the gentamicin invasion assay have to be considered. Firstly, the quantification of cluster-forming bacteria by plate-count method is less reliable due to the incomplete separation of the single bacterial cells (Barbosa et al., 1995). Secondly, as *C. pseudotuberculosis* also invades the cells as a cluster of several aggregated bacteria, the colonies grown on the agar plates might represent the number of infected cells rather than the number of internalized bacteria. Thirdly, the toxic nature of *C. pseudotuberculosis* cell surface lipid causes the death and detachment of the host cells. Detached cells, and indeed the internalized bacteria, are consecutively washed away and not accounted for in the quantification. It is therefore possible that actual *in-vivo* invasiveness of *C. pseudotuberculosis* lies higher.

7.3.1. Cytochalasin D and Colchicine

Cytochalasin D strongly inhibited the uptake of *C. pseudotuberculosis* into macrophages and lead to a significant reduction of bacterial entry in BGM cells.
The actin cytoskeleton operates crucial functions in cell motility and cytokinesis. Phagocytosis in macrophages, for instance, can be inhibited by cytochalasin D since the membrane invaginations needed for the engulfment of a particle are mediated by the actin cytoskeleton (Kwiatkowska and Sobota, 1999). The strong inhibitory effect of cytochalasin D on the internalization rates of *C. pseudotuberculosis* in the macrophages corresponds to the theory that the uptake into macrophages occurs primarily via phagocytosis.
The dose-dependent inhibition of bacterial entry seen in the BGM cells suggests that the uptake of *C. pseudotuberculosis* into epithelial cells is strongly dependent on actin microfilament rearrangement. This supports the hypothesis that the membrane pseudopods observed in the SEM pictures may be mediated by the actin cytoskeleton. Bertuccini et al. (2004) discovered that *C. diphteriae* invasion into HEp-2 cells was inhibited by cytochalasin D, and they could detect partial condensation of actin microfilaments in the area of bacterial binding by fluorescence microscopy. These findings confirmed their idea that the binding of the bacteria to specific cell surface receptors may induce a cellular signalling cascade that triggers the actin filament rearrangement, pseudopod formation and uptake via a “zipper-like” mechanism of phagocytosis. Since *C. pseudotuberculosis* invasion into the BGM cells was

also strongly impaired by cytochalasin D, the bacterium may employ a similar mechanism to invade the BGM cells. However, further investigation should be undertaken on the actin cytoskeleton dynamics in BGM cells infected with *C. pseudotuberculosis*. Examination of the BGM cells by fluorescence microscopy, for instance, would reveal cytoskeletal rearrangements and accumulation of polymerized actin beneath adherent bacteria (Hirata et al., 2004).

The treatment of the BGM cells with cytochalasin D did not totally inhibit the bacterial internalization but the inhibitory effect was more pronounced at higher doses. Alternatively, *C. pseudotuberculosis* invasion may be partially mediated by microtubules, as shown for *Campylobacter jejuni* or *Neisseria gonorrhoea* (Yoshida et al., 2003). However, treatment with colchicine, a microtubule-depolymerizing agent, did not influence bacterial entry into macrophages or BGM cells.

7.3.2. Genistein

Treatment of the macrophages and BGM cells with genistein, an inhibitor of the TPK, considerably reduced the uptake of *C. pseudotuberculosis*, especially at high concentrations. The inhibitory effect of genistein was more pronounced in the macrophages, indicating an important role for the TPK in the signalling pathway that initiates the uptake of the bacteria. Quiet analogous to FcγR-mediated phagocytosis, the uptake of *C. pseudotuberculosis* into macrophages appears to involve activation of TPK, polymerization of the actin cytoskeleton and a “zipper-like” mechanism of phagocytosis. It is unlikely, though, that *C. pseudotuberculosis* binds to cellular FcγRs, seeing that internalization into macrophages also occurs in the absence of immunoglobulins. More information needs to be gained, therefore, about the possible adhesion proteins of *C. pseudotuberculosis* and the receptors involved in the uptake into macrophages.

However, TPK-mediated signalling has also been observed upon activation of other cell surface receptors. *Y. pseudotuberculosis*, for instance, binds to cellular integrins and evokes a phosphotyrosine signalling cascade that leads to actin polymerization and engulfment of the bacteria via a “zipper-like” mechanism (Rosenshine et al., 1992; Bin Su et al., 1999). Involvement of TPK in bacterial invasion has also been reported for *H. pylori* and

L. monocytogenes (Bin Su et al., 1999; Ireton et al., 1999). Further, Hirata et al. (2002) showed that *C. diphteriae* invasion into HEp-2 cells could be inhibited by genistein. Correspondingly, *C. pseudotuberculosis* invasion into BGM cells was inhibited by genistein, indicating that the TPKs participate in the signalling cascade mediating the entry into the BGM cells. Admittedly, a significant inhibition of the bacterial invasion could only be achieved by utilizing high doses of genistein, suggesting that *C. pseudotuberculosis* may possess an alternative uptake mechanism or employ different receptors than *L. monocytogenes*, *H. pylori* or *Y. pseudotuberculosis* for their invasion into non-phagocytic cells. Overall though, the results of the experiments with genistein confirm the hypothesis of a "zipper-like" mechanism of entry of into macrophages and BGM cells since the uptake via a "trigger-like" mechanism, as seen in *S. typhimurium*, does not require TPK (Rosenshine et al., 1992).

7.3.3. Wortmannin

Wortmannin is a potent and specific inhibitor of the PI-3 kinase, which plays a key role in the signalling cascade involved in phagocytosis and seems to participate in the signalling events triggered upon binding of various invasive pathogens to their host cells. (Aderem and Underhill, 1999). *L. monocytogenes*, *H. pylori* or *Y. pseudotuberculosis*, for instance, were shown to be dependent on the PI 3-kinase for invasion into non-phagocytic cells (Ireton et al, 1999; Kwok et al., 2002; Cossart and Sansonetti, 2004). Correspondingly, the uptake of *C. pseudotuberculosis* into macrophages and BGM cells could be inhibited by wortmannin in a dose-dependent manner.

Martinez et al. (2000) showed that the invasion of uropathogenic *E. coli* seemed to require localized actin polymerization, host protein tyrosine phosphorylation and the activation of PI 3-kinase. They further demonstrated that interactions of tyrosine phosphorylated proteins with the PI 3-kinase could stimulate the activity of the enzyme. These findings correspond with the results of a study on signal transduction events triggered upon invasion of *L. monocytogenes*. Ireton et al. (1999) showed that the binding of *L. monocytogenes* protein InlB to E-cadherin induced TPK of several host proteins and a consecutive activation of PI 3-kinase by interaction of tyrosine phosphorylated proteins with the PI 3-kinase. Both authors suggested that the PI 3-kinase may transmit the phagocytic signal to the actin cytoskeleton. Possible downstream effectors of the PI 3-kinase are thereby its products, PIP_2 and PIP_3, as they are important second messengers that can affect the host cytoskeletal changes (Martinez et al., 2000), or the small GTPase Rac (Ireton et al., 1999). Bierne et al., (2000) further demonstrated that *L. monocytogenes*-mediated signalling stimulated sequentially TPK, PI 3-kinase and the activation of host phospholipase C-γ1 (PLC-γ1). Activation of PLC-γ1 would induce IP_3 and DAG, which in turn could activate PKCs and mobilize calcium from the intracellular stores. Involvement of calcium-mediated signalling during interaction of bacteria with eukaryotic cells has also been reported upon invasion of *S. typhimurium* (Bierne et al., 2000).

Wortmannin lead to a significant reduction of *C. pseudotuberculosis* internalization, indicating that PI 3-kinase plays an important role in the signalling cascade that mediates the bacterial entry. Since TPK was also required for the uptake of *C. pseudotuberculosis*, it seems likely that tyrosine phosphorylated host proteins might induce the activation of PI 3-kinase, analogous to *E. coli* or *L. monocytogenes*. However, further investigation would be necessary to determine the proteins involved in the signalling cascade and their interactions. Also, it remains unclear how PI 3-kinase transmits the signal to the actin cytoskeleton.

7.3.4. Staurosporin

Treatment of the macrophages and BGM cells with decreasing doses of staurosporin considerably enhanced the internalization rates of *C. pseudotuberculosis* in the macrophages but had no effect on the invasion into BGM cells. The dose-dependent enhancement of *C. pseudotuberculosis* internalization seen in the macrophages somewhat contrasts the results of other studies on *H. pylori* or *Y. pseudotuberculosis* showing that staurosporin, or other inhibitors of PKCs, lead to decrease in bacterial internalization (Rosenshine et al., 1992; Kwok et al., 2002). However, increasing internalization rates after treatment with staurosporin were also reported for *C. diptheriae* and *L. monocytogenes* (Wadsworth and Goldfine, 2002; Bertuccini et al., 2004,). *L. monocytogenes* was shown to produce two bacterial phospholipases C upon entry into J774 macrophages, PI-PLC and BR-PLC, which induced the production of DAG in the host and thus caused the elevation of the intracellular calcium levels (Wadsworth and Goldfine, 1998; Wadsworth and Goldfine 2002). The same group detected that the calcium-signalling was also coupled to the activation of host PKC, indicating that the PKCs contributed to the calcium release. They further found that these signalling events influenced bacterial entry and the escape of the bacteria from the phagocytic vacuole into the cytoplasm. The inhibition of PKCs with specific inhibitors lead to an increase in bacterial internalization and, at the same time, a reduced escape from the phagosomes. Their results also implicated that the bacteria appeared to communicate with the cells prior to entry, probably through secreted proteins, and that the consecutive elevation in the calcium levels prevented general phagocytosis in macrophages. Thus, the signalling events triggered by *L. monocytogenes* would decrease bacterial internalization but enhance the escape from the phagosome and intracellular survival. As the calcium signalling correlated with the escape from the primary vacuole, they further proposed that PKCs have a profound effect on later events during an infection, especially on the ability of *L. monocytogenes* to exit the phagosome.

The presented results correspond to the findings of Wadsworth and Goldfine, suggesting that *C. pseudotuberculosis* is able to employ a similar mechanism to enter macrophages, especially since the invasion experiments with *L. monocytogenes* were also performed with J774 macrophages. Analogous to *L. monocytogenes*, the PKC-signalling might negatively influence the uptake of *C. pseudotuberculosis* into macrophages but promote the escape from the phagocytic vacuole. Furthermore, the results indicate that *C. pseudotuberculosis* exhibits different invasion mechanisms for macrophages and BGM cells since staurosporin had no effect on the internalization rates in BGM cells. As proposed by Wadsworth and Goldfine, the PKCs may primarily affect the later signalling events in phagocytosis concerning the phagosome maturation and would, therefore, not influence the invasion into non-phagocytic cells. It can not be excluded, though, that the inhibitor staurosporin shows a greater affinity to macrophages than BGM cells or inhibits a different set of signalling molecules in the BGM cells. To exclude such cross-reactions, more specific inhibitors of PKCs would have to be tested.

Overall, the findings further support the hypothesis that *C. pseudotuberculosis* may actively orchestrate its escape from the phagosomes in macrophages, possibly using a similar mechanism as *L. monocytogenes*. However, future research would have to be done concerning the interactions of PKCs during invasion of *C. pseudotuberculosis* and the mechanisms used by the bacterium to escape from the phagosomes.

7.4. Conclusion

In summary, *C. pseudotuberculosis* is able to survive and multiply within macrophages and epithelial cells. The bacterium actively induces its uptake into non-phagocytic cells while the internalization into macrophages most likely occurs in a process similar to FcγR-mediated phagocytosis. In the BGM cells, *C. pseudotuberculosis* adheres in clusters, reminiscent of a “localized adherence pattern” seen in other pathogens, and induces its uptake via a ”zipper-like” mechanism of engulfment. In both, macrophages and BGM cells, the uptake is dependent on tyrosine phosphorylation, PI 3-kinase and reorganization of the actin cytoskeleton. These observations suggest a receptor-mediated entry, involving a signalling cascade that leads to the remodelling of the actin cytoskeleton and uptake via a “zipper-like” mechanism. Further, the results of this thesis indicate a possible role of the protein kinases in the uptake into macrophages and the escape from the phagosome. Overall, *C. pseudotuberculosis* invasion into macrophages and epithelial cells appears to resemble the signal-induced uptake of *C. diphteriae*, *H. pylori* or *L. monocytogenes*. It is intriguing to speculate whether *C. pseudotuberculosis* also uses similar mechanisms as *L. monocytogenes* to escape from the phagosomes and whether PLD participates in this process. However, further research would have to be undertaken on the interactions of the different signalling molecules and the cellular proteins affected by them, as well as the mechanism used by *C. pseudotuberculosis* to exit the phagocytic vacuoles.

Additionally, little is known about possible adhesion proteins of *C. pseudotuberculosis* and the cell surface receptors they could bind to. Two “non-fimbrial” surface proteins have been described in *C. diphteriae* that were shown to interact with the cell membranes of HEp-2 cells (Hirata et al., 2004). Whether such proteins exist in *C. pseudotuberculosis* and whether the bacterium interacts with the same receptors in phagocytic and non-phagocytic cells, would have to be evaluated in future research. *C. pseudotuberculosis* might also be able to trigger its uptake via several alternative mechanisms, involving different cell surface receptors and diverse signalling cascades, a feature that has been described for other invasive pathogens, e.g. *M. tuberculosis* (Gatfield and Pieters, 2000). Additionally, the lipid-rich cell wall of *C. pseudotuberculosis* structurally resembles the cell wall of *Mycobacteria,* the bacterium might, therefore, also be able to mediate its uptake through “lipid rafts” in the cell membrane.

The ability of *C. pseudotuberculosis* to survive in phagocytic cells and the importance of this attribute for the pathogenesis of CLA has been described previously (see chapter 3.3.). This study was able to demonstrate for the first time that *C. pseudotuberculosis* is also capable of invading epithelial cells. Although the number of internalized bacteria was lower than observed in other pathogens, the possibility to hide in epithelial cells even at low numbers would allow the bacterium to gain access to the host organism and evade the immune response and/or antibiotic treatment. Since invasion into epithelial cells would provide the bacterium with a more efficient way of entering the host organism and spread to distant sites, this finding might be of significance for the pathogenesis and epidemiology of CLA. Indeed, experimental infections with *C. pseudotuberculosis* indicated that the bacterium was able to penetrate freshly shorn but intact skin of sheep and establish an infection (Nairn and Robertson, 1974). Thus, the invasion of epithelial cells could represent a strategy used by *C. pseudotuberculosis* to persist in an individual animal and effectively infect other flock mates. Whether *C. pseudotuberculosis* also invades epithelial cells *in-vivo* and how this would affect the transmission and the pathogenesis of CLA, would have to be investigated in future research.

8. Index of Abbreviations

BGM cells	Buffalo green monkey cells
CLA	Caseous lymphadenitis
CR	Complement receptor
DAG	Diaglycerol
FAK	Focal adhesion kinase
FcγR	Fcγ receptor
HEp-2 cells	Epidermoid larynx carcinoma cells
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
MARCKS	Myristolated alanine-rich C kinase substrate
MOI	Multiplicity of infection
PI 3-kinase	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
TPK	Tyrosine phosphorylation

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